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A UNIQUE *CLOSTRIDIUM* SPECIES THAT PRODUCES BIOFUELS FROM MONOSACCHARIDES AND POLYSACCHARIDES

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SUMMARY

The high-energy demand and incessant fluctuations in fossil fuel price have become important issues worldwide. However, the supply of non-renewable energy resources (e.g. coal, oil, and natural gas) is currently decreasing due to the limited discovery of new mining explorations (Demirbas, 2001; de Vries et al., 2007). Because of the worldwide abundance of lignocellulosic biomass (~170 billion metric tons annually) (Klass, 1998), finding alternative fuels (e.g. biologically-generated butanol), particularly from this renewable resource, is crucial for alleviating the upcoming energy crisis and reducing the emission of greenhouse gases. Butanol represents one of the most promising fuels that can be generated from lignocellulosic biomass due to its superior chemical characteristics as compared to the more commonly used fuel, ethanol.

To screen microorganisms capable of producing butanol from lignocellulosic biomass, soil samples were collected from various locations in the Southeast Asia region. *Clostridium* sp. strain BOH3, a wild-type anaerobic microorganism demonstrating cellulolytic, xylanolytic, and solventogenic properties was isolated from paddy field soil. Its 16S rRNA gene sequence exhibited 98% identity to the sequence of *Clostridium butyricum* strain W4 (listed in Appendix). Batch culture BOH3 showed the ability to produce 4.67 g/L and 4.63 g/L of butanol from 30 g/L of glucose and xylose each. The butanol generation was further enhanced by inoculating higher amount of cells, thus improved butanol production to 7.05 g/L and 7.41 g/L, respectively. Interestingly, this culture was also able to generate significant amounts of hydrogen and volatile fatty acids from cellulose and xylan under mesophilic conditions, which is unusual because such comparable production was only observed under thermophilic conditions previously.

To further increase butanol production by culture BOH3, a combination of stepwise dosing of substrate and inoculation of high initial cell density was performed. This combination increased the production of butanol to 13.70 g/L and 10.02 g/L within 10 days from 90 g/L of glucose and xylose setups, respectively. The omission of yeast extract-peptone, which is a pricey complex nitrogen source and commonly spiked in the fermentation broth, did not affect the culture's ability to grow but produce comparable amounts of butanol.

The remarkable performance of culture BOH3 indicated the culture's potential to perform a direct conversion of biomass, such as, food waste to butanol. The food waste study showed that culture BOH3 was able to produce 14.76 g/L of butanol and 20.28 mmol of hydrogen within 6 days, from 210 g/L of dried food waste.

In summary, culture BOH3 harbors solventogenic, celullolytic, and xylanolytic properties, which have not been reported previously for wild-type cultures. Other key features of this strain that differentiate with other strains include its capability to generate higher yield of butanol as other solventogenic strains, to resist higher butanol concentration, to produce high amount of hydrogen and volatile fatty acids from cellulose and xylan at mesophilic conditions, and to perform direct conversion of food waste to butanol. Further modification of its operating conditions, like environmental conditioning and metabolic engineering, will better favor butanol production directly from lignocellulosic biomass in the near future.

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LIST OF ABBREVIATIONS

ABE	Acetone butanol ethanol
ATP	Adenosine triphosphate
CBP	Consolidated bioprocess
CO_2	Carbon dioxide
DI	Diluted-inoculation
DNA	Deoxyribosenucleic acid
FAD	Flavin adenine dinucleotide
FID	Flame ionization detector
GC	Gas chromatography
HMF	Hydroxymethyl furfural
HPLC	High performance liquid chromatography
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NOx	Nitrogen oxides
PCR	Polymerase chain reaction
pHi	Internal cells pH
pHe	External cells pH
RCA	Reinforced clostridial agar
RCM	Reinforced clostridial media
RID	Refractive index detector
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

SOx	Sulphur oxides
SO_2	Sulphur dioxide
TCA	Tricarboxylic acid
TCD	Thermal couple detector
VFAs	Volatile fatty acids
VS	Volatile solid
YEP	Yeast extract-peptone

CHAPTER 1

Introduction

1.1. Energy usage and economical development

The worldwide increase in energy usage is closely related to the rapid growth of economics worldwide. This was initiated in the 18th century during the industrial revolution which saw an era of the industrial boom, notably as a beginning of massive energy exhaustion period. The current situation of huge amount of energy demand is not solely due to the higher number of world population within years, but also with the higher annual energy consumption per capita. In order to accelerate the worldwide industrial growth, the condition was resonated with the period of oil boom in 1970s and 1980s that generated further euphoria of uncontrolled fossil fuel exploitation, such as oil, gas, and natural gas, as the cheap energy resources. This economy flourishing condition has been continuing for almost another century later (Liu et al., 2010).

The increasing and continuous energy consumption would lead to depletion of the resources of energy in the near future. Fossil fuels, currently known as the worldwide predominant resources of energy, are non-renewable and undergoing the scarcity. These problems have recently appeared more frequently with the occurrence of social and geopolitical instabilities in the fossil fuel producing countries. It has alarmed among the industrial stakeholders for the worst-case scenario of gloom period of industrial era. Condition is further complicated with the more stringent environmental regulations nowadays to combat environmental deterioration resulted from excessive consumption of fossil fuels (Boemi et al., 2010; Liu, 2010).

With the worldwide scarcity of non-renewable energy resources, the price of the fossil fuels has increased remarkably and exceeded its economical value (Liu et al., 2010). This unfavorable condition has halted the economic growth, which may hinder the improvement of the quality of human life.

1.2. Alternative renewable energy resources-lignocellulosic biomass

In order to avoid further economic recession caused by energy shortage, the search for alternative energy resources has become a necessity. One of the alternative energy resources is lignocellulosic biomass, which is abundant, relatively untapped, renewable, and more sustainable. However, little attention has been given to its exploitation due to the properties as waste, predominantly as agricultural waste that counts up to ~170 billion metric tons annually (Klass, 1998). In terms of the potential of energy generated, the forestry industries that generate lignocellulosic biomass worldwide can produce up to 1,030 quadrillion BTU/year (Demirbas, 2001; Asif and Muneer, 2007). This amount is equivalent to more than double from the world's total primary energy consumption of about 460 quadrillion BTU in 2005 (Liu et al., 2008). The high amount of energy potential extracted from the lignocellulosic biomass is further valued because this energy generation process does not involve additional carbon release to the environment. This is due to that the carbon dioxide drawn from the atmosphere for the growth of plants is re-converted into carbonaceous materials during the planting, conversion of biomass to fuels, utilization, and decomposition of the fuels. In an optimal balanced condition, carbon dioxide is simply recycled during the life span of plant growth and the generated fuels (Liu et al., 2006; Liu et al., 2008). The resulted carbon utilization is a closed carbon loop, as the solar energy and atmospheric carbon dioxide are converted to plant, derived to the lignocellulosic biomass, and converted to fuels for further utilization.

1.3. Energy exploitation from the lignocellulosic biomass

A sustainable fuel generation comes through a sustainable raw material, process, and technology. Energy extraction can be performed through various energy-generating processes that are implemented in energy-generating technology, such as physical, chemical, thermal, biological, or the combined technologies (Magnani et al., 2007; Gomez et al., 2008). Till date, physical, chemical, and thermal processes for energy exploitation from the lignocellulosic biomass have shown attentive results due to the high rate and high yield of fuels production in the laboratory- and pilot-scales. However, an up-scaling to implement the abovementioned processes into the technological level has shown a hindrance resulted from the high energy consumption and high production of chemical by-products (Hendriks and Zeeman, 2009). This has escalated the fuels production cost, embarked an uneconomically feasible technology, and defeated the purpose of sustainable energy generation process. Therefore, much attention has been given to the cost-effective, well-controlled, sustainable biotechnology, as an implementation of a biological process to extract energy.

Biological process was initially underestimated, due to the difficulty to control and maintain the process, as it employs living cells, such as microorganisms in the process. However, the updated technologies that can better understand and control microorganisms' growth conditions have aligned the bioprocess as a promising solution to the sustainable conversion of lignocellulosic biomass into energy (Magnani et al., 2007; Gomez et al., 2008). The mild conditions in the biological process are paramount to decrease the amount of energy consumption and chemical by-products as compared with other processes. These superiorities of biological process or bioprocess has attracted more scientific attention for lignocellulosic biomass conversion to fuels (Magnani et al., 2007; Gomez et al., 2008).

All processes and technologies offer the advantages and disadvantages, including the biological process for biofuels production. Some drawbacks of biological process still exist, such as the low production rate and yield, which may hinder the technological application of biological process (Taherzadeh and Karimi, 2008). However, all of the shortcomings from physical, chemical, and thermal processes can be overcome by biological process. This has aligned the direction of the search for an improved biological process that can be further up-scaled into a robust technology and has a comparable performance as physical, chemical, or thermal process.

1.4. Potential biofuels-biobutanol

As an alternative route for biofuels production through biological process, various types of biofuels have been globally recognized, such as bioethanol, biodiesel, biomethane, biohydrogen, etc. These fuels have distinct characteristics according to their forms, energy densities, and physical or chemical characteristics.

Among the abovementioned biofuels, biobutanol is one of the promising fuels that can replace bioethanol as the most currently used liquid fuels worldwide (Lee et al., 2008). In comparison with ethanol, butanol has lower heat of vaporation, higher blending ratio with gasoline, higher energy content, and lower gaseous emission after combustion (particularly NOx and SOx). Butanol is also less corrosive, easier to transport, less hydroscopic, and more miscible with gasoline or diesel fuel. Most importantly, it can avoid the engine modification, thus eliminating the engine retrofitting cost (Dürre et al., 2008; Lee et al., 2008). These advantages of butanol over ethanol will be further enhanced when it could be produced through biological process, which has been described previously as a more sustainable and environmental friendly process.

In term of world trading, annual worldwide butanol production is ~1.32 billion liters. The current selling price of butanol is about US$ 0.97 per liter in comparison with US$ 0.52 per liter for ethanol, with annual projected market expansion of 3% (Dürre, 2008). Even the production of butanol from biological route has been overcompeted with the petrochemical route since 1960s, the current high demand of butanol and stricter environmental regulation have further revived the idea to generate butanol from non fossil fuel. Currently, over a dozen of butanol production plants have been built or rebuilt, and some are in the planning stage in China (Ni and Sun, 2009). Some of the plants use starchy materials as the substrates, such as corn, cassava, and potato powder, in order to achieve higher fermentation performance that results to the higher butanol production. However, these substrates are considered as food staples that might induce the food price hike due to the competition of substrates for fuel and food (Gu et al., 2011). Thus, the cheaper starchy material or lignocellulosic biomass conversion to butanol through a biological process would be the utmost milestone to achieve sustainable biofuels production in near future (Dürre et al., 2008).

1.5. Butanol production through anaerobic biological process

Butanol production through biological process is an anaerobic fermentative process. Substrate conversion to butanol is catalyzed by the enzymes synthesized from bacteria in the absence of oxygen and is coupled with the production of hydrogen, carbon dioxide, volatile fatty acids (VFAs), and growth of bacterial cells. Acetone and ethanol are also produced along with butanol, as solvents, in less significant amounts (Lee et al., 2008). However, the majority of the substrates for butanol production are monosaccharides, which enable the microorganisms to be

involved only in a simple fermentation process. Currently, all of the strains for butanol production are limited to *Clostridia* genus, such as *Clostridium acetobutylicum, Clostridium beijerinckii,* and *Clostridium pasteurianum* (Kumar and Gayen, 2011). All of these *Clostridia* species are mesophilic, rod-shaped, spore-forming, gram positive, strict anaerobic bacteria, with the preference of free monosaccharides as the substrates for the butanol production (Dürre, 2005). The microbes which are able to directly convert polysaccharide structure of cellulosic substrate, such as lignocellulosic biomass to butanol still remain unidentified. Given that the free monosaccharides are limited in nature, it is therefore desirable to screen a robust culture, capable of directly converting the abundant lignocellulosic biomass to butanol.

1.5.1. Preliminary processes prior to the fermentation process

In order to convert the abundant lignocellulosic biomass to butanol, several preliminary processes are essential prior to the fermentation process, such as delignification of lignin and hydrolysis of polysaccharides to monosaccharides. Most of the current delignification processes are conducted through physical, chemical, thermal, or the combined methods, which are unsustainable and costly. Other than the harsh condition to perform a delignification process, the delignified products, such as furfuric acid, p-coumaric acid, hydroxymethyl furfural (HMF), syringaldehyde, glucuronic, and ferulic acid, are also inhibitory for microorganisms in the fermentation process (Ezeji and Blaschek, 2008). Thus the delignification process generally requires the removal of these inhibitory compounds, mostly by overliming method, prior to the hydrolysis and fermentation processes (Ranatunga et al., 2000).

On the other hand, hydrolysis process is mostly performed through the addition of exogenous fungi enzymes. Hydrolysis process aims to reduce the

crystallinity and hydrolyze polysaccharides to monosaccharides. For instance, cellulose is hydrolyzed to six-carbon monosaccharides, predominantly glucose, and trace amounts of galactose and mannose. Xylan is hydrolyzed to five-carbon monosaccharides, predominantly xylose, and trace amount of arabinose (Sun and Cheng, 2002). The produced glucose and xylose are later fermented by solventogenic *Clostridia*. However, the high cost of enzymes contributes significantly to hike the production cost of the biofuels.

In summary, these two separate preliminary processes are not economically affordable and technically feasible as an approach for lignocellulosic biomass conversion. A direct conversion of lignocellulosic biomass to butanol in the less harsh condition that combines delignification and hydrolysis processes is termed as consolidated bioprocess (CBP) (Lynd et al., 2005; Yang et al., 2009). It is therefore likely to perform a single step process, which combines delignification, hydrolysis, and fermentation processes of lignocellulosic biomass to butanol, with a comparable performance to an up-scaled technology level. In this study, "direction conversion" is used to describe the process to all of the substrates unexceptionally, which include the lignocellulosic biomass and non-lignocellulosic biomass, such as pure cellulose, pure xylan, food waste, etc., in which there will be lesser extent of delignification process as compared to lignocellulosic biomass.

The efficacy of the direct conversion process is largely impacted by the difference of operational conditions between delignification, hydrolysis, and fermentation processes. Delignification process needs low pH and high pressure or temperature to delignify the lignocellulosic biomass, while hydrolysis needs neutral pH to perform (Lynd et al., 2005). For fermentation process, different conditions will result in different products of biofuels, such as low pH for butanol production and

neutral pH for hydrogen production (Dürre, 2005). Thus, the optimization for a condition that encompasses delignification, hydrolysis, and fermentation processes, would be another paramount for the exploration of direct conversion process.

1.5.2. Cultures involved in direct conversion

For hydrogen and ethanol productions, some pure strains have been known to have direct conversion capability. Some of the direct conversion performers are *Clostridium thermocellum* and *Anaerocellum thermophilum* DSM6725 for lignocellulosic biomass (Lynd et al., 2005; Kataeva et al., 2009). Cultures *Anaerocellum thermophilum* Z1320 and *Caldicellulosiruptor saccharolyticus* DSM8903 are able to directly convert cellulose and xylan into ethanol, lactic acid, acetic acid, hydrogen, and carbon dioxide (Svetlichnyi et al., 1990; Mladenovska et al., 1995). To the best of our knowledge, little is known on the isolated pure strains or consortia that are able to perform direct conversion to butanol, from cellulose, xylan or lignocellulosic biomass. The search for a proper substrate and robust strains is important to achieve direct conversion of substrates to butanol. In the initiation stage, the exploration to get an isolate that harbors cellulolytic, xylanolytic, and solventogenic properties would be of great significance to the achieve the goal.

1.6. The mechanism of butanol production

The mechanism of butanol production largely depends on the initiation stage by the acidogenic process, in which acetic acid and butyric acid are the predominant produced acids. This stage is also the exponential phase of cell growth, coupled with exponential generation of hydrogen and carbon dioxide gases. The presence of produced acids decreases pH, thus creates unfavorable environment for the bacteria. The decrease of pH to ~4.0–5.0 would generate a self-defense mechanism for the

bacteria, transform its vegetative cell stage to sporulation stage. Once the sporulation stage starts to be onset, cells would re-assimilate the acetic acid and butyric acid in the fermentation broth to the neutral compounds, such as solvents. The shift of acidogenic to the solventogenic stage is a typical and unique mechanism of solventogenic *Clostridia* (Dürre, 2005). The incremental of butanol production up to the butanol toxicity limit is decoupled with the cell growth. However, the produced butanol starts to cease once the substrate depletes and the butanol toxicity limit has been achieved (Dürre, 2005).

Butanol toxicity is an inevitable limitation for butanol production through bioprocess. Addition of substrate does not improve the maximum yield of butanol, due to the toxic effect of butanol to the culture. Although some genetic modification studies have shown the improvement of butanol production up to 17.8 g/L by performing solR gene down-regulated modification (Lee et al., 2008), the highest final concentration of butanol by wild-type *Clostridia* has never exceeded ~15 g/L (Lee et al., 2008). Lipophilicity of butanol to the cell membrane is found to be more destructive than acetone and ethanol, as it disrupts the phospholipid components of the cell membrane, thus increases the membrane fluidity (Bowles and Ellefson, 1985). The increase of membrane fluidity causes membrane destabilization and disruption of membrane-associated functions such as transport process, glucose uptake, and membrane-bound ATPase activity (Bowles and Ellefson, 1985). The cell growth starts to be inhibited when there is presence of 7.4–11.1 g/L of butanol in the fermentation broth (Moreira et al., 1981; Woods, 1995). In order to minimize this toxicity, it is a common practice to install a continuous fermentation reactor parallel with the continuous separation of butanol.

The phenomenon of butanol toxicity which hinders the further incremental of butanol final concentration has not only been recognized in the laboratory-scale, but also in industrial-scale. During the cell density depletion in the reactor, the butanol production ceases. This occurrence shows the necessity to maintain high cell density in the reactor, without sacrificing the butanol production (Dürre et al., 2008; Lee et al., 2008). Therefore, attached biological growth of the cells or inoculation of high-density cells, coupled with the parallel butanol removal, would be an appropriate configuration to achieve higher final concentration, rate, yield, and productivity of butanol. Addition of exogenous yeast extract-peptone (YEP) as the complex nitrogen source for a more flourished cell growth has further increased the butanol production cost (Parekh et al., 1999; Campos et al., 2002). The process manipulation by performing a high-density cells system might subdue the necessity of YEP addition, thus further suppresses the production cost of direct conversion to butanol. This effort will further place the application of butanol production from lignocellulosic biomass in an economically affordable process, implementable, environmental friendly, and more sustainable.

1.7. Problem statement

Most of the solventogenic *Clostridia* do not have distinct cellulolytic and xylanolytic properties. There was a study for *Clostridium acetobutylicum* that has cellulolytic activity. However, the detail of its cellulolytic activity has never been reported, in term of performance of direct conversion of cellulose or xylan to fermentation products (Gal et al., 1997). The searching for a wild-type isolate possessing high cellulolytic and xylanolytic activities will be promising for a direct conversion of cellulose, xylan, or lignocellulosic biomass to butanol or other energy-rich products, such as hydrogen, preferably performed in the mesophilic condition due

to its lower energy input in such process. Genetic engineering of the microorganisms is less preferable, due to the commonly found instability for these genetically-modified cultures during the fermentation batches. In addition, these genetically-modified cultures could also be present in the residue of the fermentation process, which might generate an unknown environmental impacts for the ecological balance (Levin and Stewart, 1977; Lewin, 1982).

In order to achieve high concentration of butanol, various conditions are set to achieve higher final concentration, yield, rate, and productivity from the wild-type microorganisms. As described previously, the depletion of cell density during the solventogenic stage hinders the high butanol production. This can be overcome by maintaining the high cell density, which may help to maintain or improve the process performance. From the substrate dosing perspective, the common bulk dosing method usually performed in the fermentation process may not be suitable for wild-type *Clostridia*, because the production of excessive acids may result in a time-inadequacy for the premature culture to re-assimilate the acids to butanol (Lee et al., 2008). Furthermore, provision of the high concentration of substrate needs a highly concentrated substrate to be dispensed in the fermentation system, which is not technically feasible. Addition of yeast extract-peptone (YEP) as the pricey nitrogen source, further escalates the butanol production cost. Hence, a modified process to achieve comparable amount of butanol with the absence of YEP would be a breakthrough to achieve an economically affordable butanol production. This can be approached by modifying substrate-dosing method and maintaining inoculation method for the high cell density even with the absence of YEP addition (Parekh et al., 1999; Campos et al., 2002).

A suitable wild-type microorganism harboring cellulolytic, xylanolytic, and solventogenic properties is the first attempt to achieve a direct conversion of lignocellulosic biomass to butanol. The employed culture can be later optimized to achieve a better fermentation process, specifically the high yield and rate of butanol production. A further investigation to better align the implementation of a full-scale butanol production is the selection of the most appropriate substrate. One of the most promising substrates is the biomass, specifically food waste that is a starchy waste material. Food waste does not compete with the food supply chain, as compared with cassava, sago, or corn (Grobben et al., 1993; Gu et al., 2009; Thang et al., 2010). Furthermore, there are limited studies about direct conversion of food waste to butanol. It is therefore important to investigate the reliability of abundant food waste as a potential substrate candidate for direct conversion to butanol. Hence, the discovery of a suitable wild-type microorganism, determination a proper selected substrate, and optimization the process modifications, are the important milestones in scientific finding to achieve higher butanol production in an economically affordable and technically feasible approach.

1.8. Objectives

This study was aimed to discover a wild type cellulolytic, xylanolytic, and solventogenic isolate, which can be applied as a potential candidate for a direct conversion of lignocellulosic biomass to butanol in the future. The isolate was later further examined for the cellulolytic, xylanolytic, and solventogenic performances by checking its ability to produce hydrogen and butanol in a direct conversion process. An initial study to employ the isolate to produce butanol by combination of the modified substrate feeding simultaneously with the high cell density inoculation, was hypothesized to implement the up-scaling from the laboratory-scale to the bioreactor-

scale. This method will enhance an understanding to achieve efficient butanol production process, which can subdue the common practice by adding YEP as the costly complex nitrogen source.

The study to determine appropriate substrate for direct conversion to butanol, such as food waste, was also explored. Food waste is a cheap, abundant starchy waste material but unaffected with the food supply chain. It thus can be proposed as the substrate for butanol production in industrial-scale application.

The specific objectives of this study are to:

1. Explore the diversity of microorganisms collected from natural environments and characterize their butanol-producing potential.
2. Characterize and isolate the novel wild-type butanol-producing microorganisms from monosaccharides and polysaccharides.
3. Optimize the substrate dosing method and high-density cell inoculation approach to achieve high concentration of produced butanol from the wild-type isolate, with the absence of complex nitrogen supplement.
4. Determine the feasibility of direct conversion of food waste to butanol using the obtained isolate.

1.9. Organization of the thesis

This thesis is divided into six chapters as follows, defining the in-depth of the particular study that contributes to meet the study objectives. For chapter 3-5, each chapter contains individual materials and methods, results, discussion, and conclusion parts, which are specific to the area of study.

Chapter 2 discusses the overall knowledge that supports the studies as in following chapters. The current stage of butanol production is highlighted, including

wild-type strains that have solventogenic properties, the maximum butanol that can be produced, and various metabolic engineering processes that have been performed to improve butanol production. Delignification and hydrolysis processes as the prerequisites of the solventogenic process are presented to give a better insight for the direct conversion to butanol. Process modification to support the study that has been proven to improve butanol production is also expounded. Information about downstream process to butanol separation is briefly discussed, as current study does not involve the downstream separation process. However, this information is useful to accomplish a complete picture of butanol production from the lignocellulosic biomass.

Chapter 3 discusses about isolate BOH3, a wild-type *Clostridium* which showed cellulolytic, xylanolytic, and solventogenic properties. Although this isolate did not show butanol production directly from cellulose or xylan, however, this unique feature reveals a potential for the direct conversion of those abovementioned polymeric substrates to butanol in the future. A comparable amount of produced hydrogen from a direct conversion of cellulose and xylan in a mesophilic condition was also observed from this study, in addition to its strong solventogenic properties in monosaccharides setups. This also suggests another possibility for hydrogen production from cellulose and xylan, other than butanol from monosaccharides. Butanol toxicity study was also conducted to check the resistance of culture BOH3 to the presence of butanol.

In an attempt to improve butanol production by culture BOH3, chapter 4 presents an intermittent substrate dosing approach and application of high cell density

inoculation. As compared to the common bulkdosed substrate, the stepwise dosing of substrate has shown an efficient fermentation process. It was assessed by the high final concentration, yield, rate, and productivity of the butanol production, even without the introduction of costly yeast extract-peptone (YEP) and pH control system. Higher cell density application was found to be able to shorten the fermentation time, however, butanol tolerance level was not significantly altered, due to the butanol toxicity limit. Interestingly, higher amount of hydrogen was generated from the utilized substrate, which indicated a fermentation pathway shifting from butanol production to hydrogen production.

Chapter 5 discusses about the direct conversion of food waste to butanol by culture BOH3. Food waste is an abundant, costless, and starchy waste material, which does not compete with the food supply chain. Its potential to perform direct conversion to butanol was explored. To the best of our knowledge, there is limited information of strains which can perform a direct conversion to butanol. Coping with that fact, food waste was found to show promising results for the possibility of up-scaling in an industrial-scale of direct conversion to butanol.

This last chapter summarizes the major findings of this study, the overall performance of culture BOH3, in term of its cellulolytic, xylanolytic, and solventogenic properties. The optimization of high butanol production yield through modification of inoculation and substrate dosing approaches are also discoursed in this chapter. The promising application about the possibility of direct conversion of food waste to butanol by culture BOH3 is also expounded in this chapter.

CHAPTER 2

Literature review

2.1. Energy demand, resources shortage, and environmental concerns

The development of civilization and higher expectation of comfort in life has driven a continuation of human development. However, this condition is also coupled with the requirement of high-energy demand. Hence, the higher annual energy consumption per capita, followed by the higher number of world population, has risen the concern to gain energy with high abundance but at low cost. Currently, the annual worldwide amount of energy demand (~370 Exajoules) is still relying on the fossil fuels, in which 95% proportion is predominated by coal, oil, and natural gas (Chow et al., 2003). Many countries nowadays still depend on fossil fuels as the main resources of energy (Chow et al., 2003). Back to the history, fossil fuels have empowered the world since James Watt's invention on steam engine. This has initiated the massive world industrialization since 1769, thus alleviating the economic growth (Nuvolari, 2004). However, these non-renewable energy resources are considered unsustainable, as its supply will diminish in future (Asif and Muneer, 2007).

A study (Höök et al., 2009) concluded that the decline in worldwide crude oil production started in the end of 2005. Condition is worsened with the prediction that annual global oil production will decline from the current 25 billion barrels to approximately 5 billion barrels in 2050 (Bon, 2005). The geopolitical instability in oil-producing countries recently, also further hiked the world oil price (Kesicki, 2010). Simultaneously, the more stringent environmental issues currently have discouraged the excessive use of non-renewable energy resources, instead has

encouraged the renewable energy resources. This issue has also been addressed in the international fora, recently in the Copenhagen Summit (December 2009) and Cancun Summit (November 2010). These two summits have ratified the global consensus due to the concern on climate changes that is contributed by the fossil fuels. Fossil fuels burning was considered as generator for enormous amount of carbon dioxide emission to the atmosphere (Wilks, 2009; Woodward, 2009; Waxman, 2010).

2.2. Renewable energy resources

There are various types of renewable energy resources that currently can be selected as the alternatives for non-renewable energy sources, such as photovoltaic, wind, geothermal, hydraulic, and biomass (Dincer, 2000). With the specific characteristics of renewable energy resources, the application is also at different levels in every country. This is due to the specific conditions and situations in the particular country. Some of the considerations include the annual sunlight intensity, annual windspeed, the availability of water body that has to meet certain criterion of flow rate, the abundance of biomass, the presence of volcanic activity, etc. However, some considerations can be evaluated to assess the feasibility of the renewable energy resources, specifically from the economical and environmental impacts perspective (Espey, 2001).

As shown in Table 2.1., biomass requires the highest amount of land to produce similar amount of energy compared with other renewable energy resources. Inversely, geothermal needs the least amount of land requirement, although the highest energy output to input can be gained. In term of production cost, photovoltaic is considered as the most expensive one and hydraulic power lies as the cheapest for its production cost. The lifetime of all the non-renewable energy-generating infrastructures are similar (30 years), except geothermal which is 20 years. Table 2.2

and 2.3 present an assessment that is based on the input and output of materials to generate energy from various renewable resources.

Table 2.1.
Economical consideration for various renewable energy resources*

Energy resources	Land requirement (km^2)	Energy input : energy output	Production cost (US$/kWh)	Technical lifetime (years)
hydraulic	75.0	1:24	**0.02**	**30**
biomass	2,000.0	1: 7	0.06	**30**
wind power	95.0	1:4	0.07	**30**
photovoltaic	28.0	1:7	0.25	**30**
geothermal	**0.3**	**1:48**	0.07	20

* *Based on the calculation to produce 1 billion kWh/year*
Ref: (Pimentel et al., 2002)

Table 2.2.
Input of energy/materials from various renewable energy resources*

Energy resources	Input		
	CED (MJ)	iron ore (g)	bauxite (mg)
hydraulic	0.14	**2.00**	16.00
biomass	**0.09**	2.50	34.00
wind power	0.12	3.30	4.80
photovoltaic	1.50	3.30	1,200.00
geothermal	0.54	3.20	**4.70**

* *Based on the calculation to produce 1 billion kWh*
Ref: (Pehnt, 2006 ; Cherubini et al., 2009)

An investment of energy that must be allocated to generate the renewable energy is an important justification, despite the infrastructures are also aimed for energy generation. Based on comparing the ability of the infrastructures to produce 1 billion kWh, photovoltaic is considered the highest energy-consumer, whereas biomass is the lowest one. In terms of the material balance, hydraulic power consumes the highest amount of iron ore whereas geothermal utilizes the highest amount of bauxite. A good energy-generator system requires the least amount of

energy and material consumption in order to achieve the positive gaining of energy generation. On the other hand, the environmental impacts from various renewable energy generators must also be considered through the output of environmental pollutants. As presented in Table 2.3, it is clearly expounded that biomass is considered as renewable energy resource that enable to combat the potential of greenhouse gas emission, due to its negative value in emitting CH_4 and N_2O. Other parameters are considerably similar for all renewable energy resources, except that wind power also has less amount of pollutants emission. Photovoltaic is relatively moderate in term of pollutants emission. This is due to that sunlight as the energy source for photovoltaic is considerably worldwide abundant. Notably, the subtropical countries receive less amount of sunlight compared to tropical countries (Cherubini et al., 2009). In terms of economical feasibility, the wind power is considered as the most feasible choice due to its lowest production cost, the least impacts on the environmental pollution, and the high abundance. However, the lagging of wind power application is due to its specificity to the geographical location, as the minimum wind speed is required to achieve a sustainable wind power system (Datta and Ranganathan, 2002). Photovoltaic is also specific to the certain locations that has adequate sunlight intensity over the year (Richards, 2006). Through the comparison of the energy input and output among the above five renewable energy resources, biomass, wind, and photovoltaic are listed as the top three prospective candidates. Furthermore, the presence of biomass is less affected from the geographical perspective, as biomass is always available in any parts of the regions worldwide (Pehnt, 2006). Due to this assessment, biomass among other energy resources can be considered as the most feasible option of renewable energy resource in the near future.

In summary, biomass is considered as the most feasible option for the renewable energy source compared with the other four options due to: (i) low energy input requirement to exploit energy generation from biomass. (ii) high greenhouse gas emission that can be eradicated. (iii) the lowest energy production cost in the long term run. (iv) high abundance in all regions worldwide.

2.3. Lignocellulosic biomass as a renewable energy resource

Biomass is highly recognized as a promising resource for energy production, because it stores chemical energy through photosynthesis process. Photosynthesis is the process in which lignocellulosic biomass converts CO_2 to energy-rich organic compounds, with the presence of sunlight. Thus solar energy is captured and stored in the form of chemical energy (Demirbas, 2001). The stored chemical energy can be further converted into more types of energy-rich products through various technologies (Demirbas, 2001).

Specifically, lignocellulosic biomass is spread over and world-widely abundant but currently with low utilization rate. The most predominant regions for lignocellulosic biomass are in Asia, Africa, and Latin America, as shown in Fig 2.1 (Demirbas, 2009). In Asian countries, Indonesia, China, and India are the galore for the lignocellulosic biomass potential (Fig 2.2.).

Table 2.3.
Output of pollutants from various renewable energy resources*

Energy resources	Output												
	GHG	**CO_2**	**CH_4**	**N_2O**	**SO_2**	**CO**	**NO_x**	**NMHC**	**particulates**	**HCl**	**NH_3**	**benzene**	**benzo(a)-pyrene**
	(g CO_2-eq/MJ)	**(g)**	**(mg)**										**(µg)**
hydraulic	**0.50-10.00**	13.00	29.00	0.70	**28.00**	**74.00**	49.00	11.00	**31.00**	0.20	**0.06**	0.05	**0.30**
biomass	15.00 – 65.00	11.00	**-19,763.00**	**-743.00**	368.00	723.00	575.00	166.00	38.00	**0.10**	1,619.00	**0.02**	0.40
wind power	1.00-10.00	**10.20**	24.10	0.20	39.50	96.80	**31.10**	26.10	42.20	0.20	0.06	0.05	**0.30**
photovoltaic	15.00–40.00	99.00	220.00	1.90	288.00	141.00	340.00	20.00	119.00	2.90	0.71	0.09	1.40
geothermal	2.00-10.00	37.80	103.40	2.60	61.60	208.00	188.90	**0.00**	35.40	1.10	0.70	0.05	**0.30**

* *Based on the calculation to produce 1 billion kWh*
Ref: (Pehnt, 2006 ; Cherubini et al., 2009)

Table 2.4. Production cost from various renewable energy resources

Energy resources	Unit cost (US$/kWh)			
	Current	Short term (2010-2020)	Medium term (2030)	Long term (2050)
wind	**0.05-0.13**	**0.03-0.08**	**0.03-0.05**	**0.03-0.09**
photovoltaic	0.25-1.24	0.25-0.40	0.15-0.30	0.07-0.25
biomass	**0.05-0.10**	**0.03-0.08**	**0.03-0.04**	**0.03-0.10**

Ref: (de Vries et al., 2007)

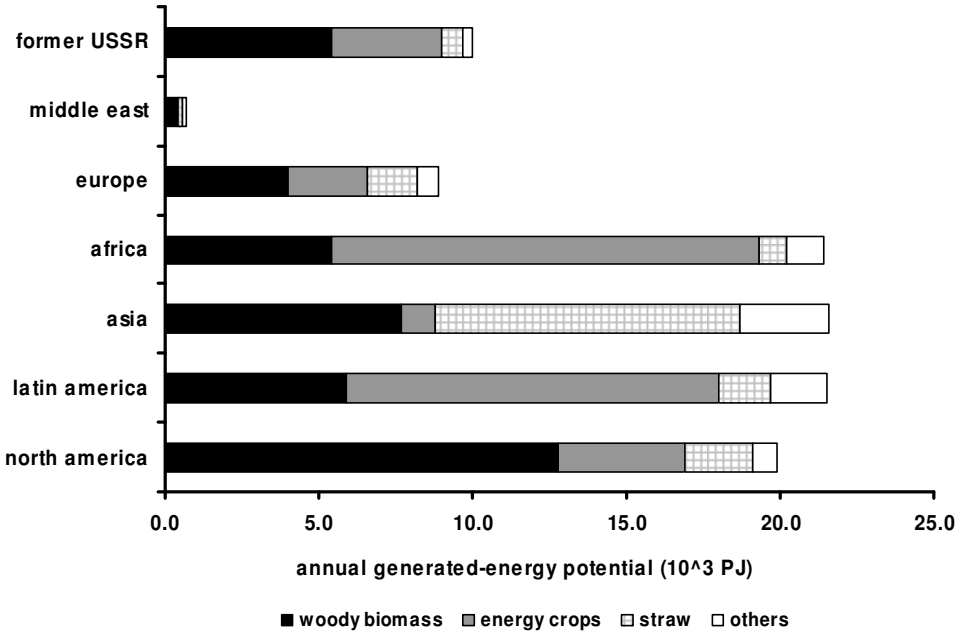


Fig. 2.1. Global distribution of lignocellulosic biomass *(Demirbas, 2009)*

Currently, lignocellulosic biomass is considered as one of the relatively untapped renewable energy resource on earth. Annual world production of lignocellulosic biomass is ~146 billion metric tons, in which some farm crops and trees can produce up to 49 metric tons per hectare (Demirbas, 2001). In developed countries, biomass utilization is considered low (~3%) from the total primary energy usage. However, 50% of world population predominantly living in the villages of developing countries is originating 35% of their primary energy source from biomass.

Thus, overall worldwide biomass conversion to energy is still low (~14%) (Demirbas, 2001).

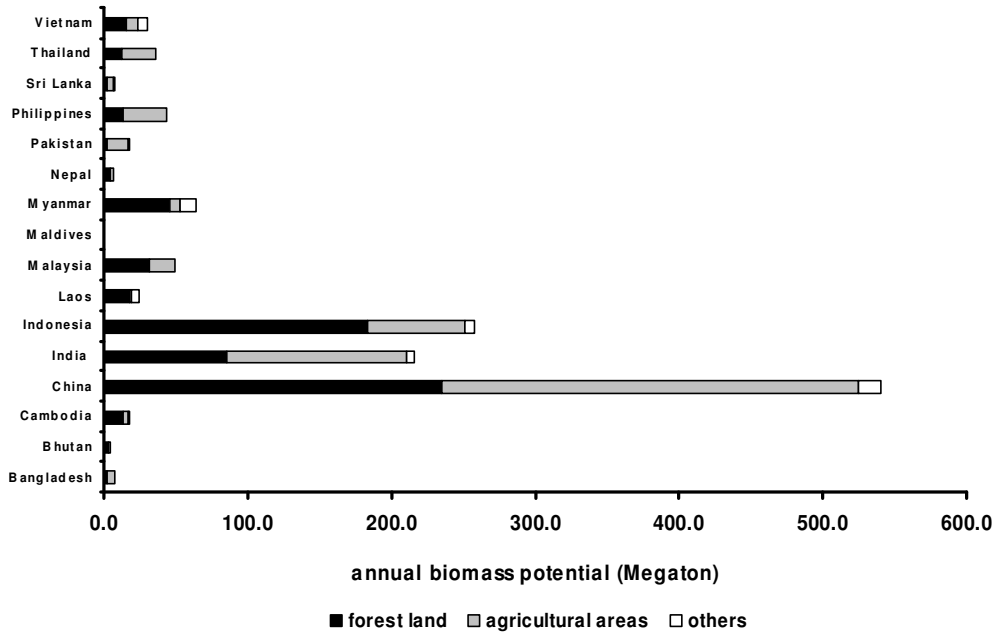


Fig. 2.2. South East and South Asia distribution of lignocellulosic biomass
(Koopmans, 2005)

There are four major classifications of lignocellulosic biomass on earth that can be converted as energy-producer, which are wood residues (64%), municipal solid waste (24%), agricultural residues (5%), and dedicated energy crops (7%) (Demirbas, 2001). They are mainly generated from the forestry industries which include paper mills, sawmills, and furniture manufacturing. Municipal solid waste is the next largest, followed by agriculture residues and dedicated energy crops. This proportion is due to the abundant amounts of harvests from the plantation (Hoogwijk et al., 2003). As the substrates for the alternative energy resource, lignocellulosic biomass has another advantage as it does not compete with the food source. The abundance of lignocellulosic biomass which is considered as agricultural waste, does not affect the

food price. This promising feature of lignocellulosic biomass is beneficial according to the current situation of food price hiked, due to the competition of substrate for fuel and food (Johansson and Azar, 2007; Tilman et al., 2009; Rathmann et al., 2010).

2.4. Physical and chemical structures of lignocellulosic biomass

Lignocellulosic biomass has a complex structure as plant cell wall consists of cellulose, hemicellulose, and lignin. Cellulose is an insoluble compound, with the linear structure of unbranched homopolysaccharide that consists of glucose subunits linked via β-1,4 glycosidic linkages. Hemicellulose is non-cellulosic polysaccharides, mostly consists of xylan, with trace quantities of mannan and glucan. Cellulose and xylan are main sources of energy in the lignocellulosic biomass, with cellulose as the predominant part (40-50% by dry weight basis) followed by xylan (20-25% by dry weight basis) (Hopkins, 1999). Lignin incorporates the structure between cellulose and xylan in lignocellulosic biomass (Claasen et al., 1999).

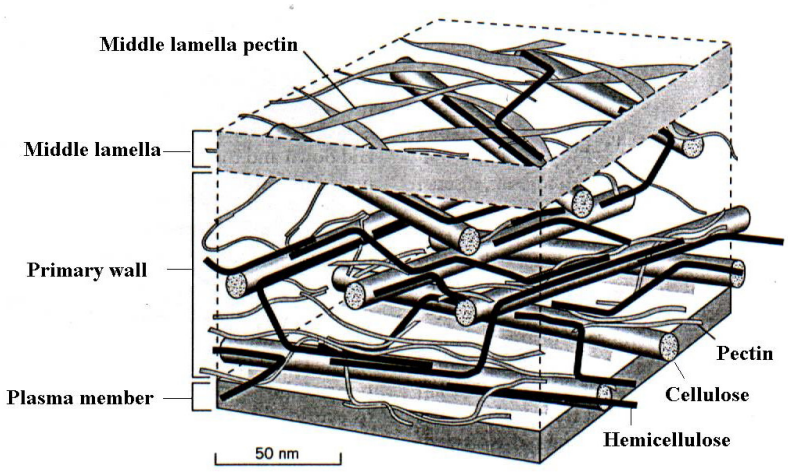


Fig. 2.3. Cellulose, xylan, and lignin structures in lignocellulosic biomass *(Hopkins, 1999)*

The presence of xylan and lignin contributes to the limited accessibility for the cellulose conversion by physical, chemical, thermal, or biological process. Cellulose

itself is more preferable rather than xylan to be converted to fuels, due to the high proportion of cellulose and the preference of fermentative microorganisms to utilize hydrolysate of cellulose (glucose) instead of hydrolysate of xylan (xylose) (Hopkins, 1999). The complexity of the structure varies in different plant, with respect to the size and organelle configuration. For instance, mesophyll structure consists of thin and poorly lignified walls, which makes the enzymes infiltration easier. Other structure may have more complicated structure, which requires the deeper penetration of enzymes to the cell walls from the inside surface out through the secondary wall. The more delicate and complex structure requires high accessibility for the enzymes to infiltrate. Thus, the efficiency for the delignification and enzymatic hydrolysis processes would decrease. Table 2.5. lists the composition of cellulose, xylan, and lignin in various types of lignocellulosic biomass.

Table 2.5. Composition of cellulose, xylan, and lignin in various types of lignocellulosic biomass

Lignocellulosic biomass	(% dry weight basis)		
	Cellulose	Xylan	Lignin
corn stover	37.5	22.4	17.6
corn fiber	14.3	16.8	8.4
oil palm empty fruit bunch	50.4	21.9	10.0
pine wood	46.4	8.8	29.4
poplar	49.9	17.4	18.1
wheat straw	38.2	21.2	23.4
switch grass	31.0	20.4	17.6
office paper	68.6	12.4	11.3

Ref: (Umikalsom et al., 1997; *Claassen et al., 1999; Mosier et al., 2005)*

2.4.1. Cellulose

A distinctive and unique feature of cellulose is its crystalline structure in comparison with other polysaccharides. Other than crystalline structure, cellulose also contains the amorphous regions. The crystallinity of cellulose is determined by the

relative amounts of crystalline and amorphous regions, in which ~66% of cellulose area is in the crystalline region (Chum et al., 1988). Enzymatic hydrolysis process to hydrolyze and solubilize cellulose shows a higher efficacy for the amorphous portion instead of the crystalline region of cellulose. It is therefore expected to decrease the crystallinity of cellulose that would in turn increase the digestibility of lignocellulosic biomass (Fan et al., 1980).

Cellulose is naturally synthesized as individual molecules that undergo at the site of biosynthesis. The overall cellulose fiber is self-assembled from microfibrils, which contain multiple protofibrils. Protofibrils consist of approximately 30 individual cellulose molecules (Brown and Saxena, 2000). These protofibrils are mostly independent in structure and bounded weakly in hydrogen bond (Laureano-Perez et al., 2005).

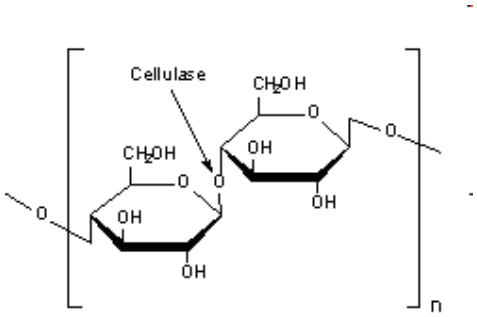


Fig. 2.4. Cellulose chemical structure *(Hopkins, 1999)*

Purified cellulose that is commonly used for scientific studies varies considerably in term of their fine structural features. Thus, the choice of cellulose types for such studies undoubtedly affects the obtained results. Microcrystalline cellulose (e.g., Avicel and Sigmacells) are nearly pure cellulose, in which the dilute acid treatment was initially conducted during the preparation to further remove the xylan and amorphous regions of the cellulose (Brown and Saxena, 2000). The

difficulty for the hydrolysis study due to the variability of structural complexity of pure cellulose as insoluble substrate has led to the wide use of carboxymethylcellulose (CMC). CMC is highly soluble cellulose ether and has been widely used as a model of cellulose and cellulase production, including endoglucanase, exoglucanase, and β-glucosidase productions (Fields et al., 1998). Due to the simplified structure of cellulose, the use of CMC as the model for the substrate has weakened the meaning of the cellulolytic ability. Many microorganisms have been investigated for their properties to utilize CMC, instead of cellulose as the carbon source. From this perspective, the presence of microorganisms, especially bacteria which can degrade crystalline cellulose as a more complex substrate than CMC, would be an avenue for the discovery of cellulolytic bacteria (Fields et al., 1998).

2.4.2. Hemicellulose as xylan

Hemicellulose as xylan enclaves the cellulose from hydrolyzing enzymes attacks. Its chemical structure has various branches with several short lateral chains, and consists of different monosaccharides with lower molecular weight than cellulose (Fengel, 1984). Some of the monosaccharides of hemicelluloses are trace amounts of arabinose and mannose, with of xylose as the predominant part. In terms of hydrolysability of various components of hemicellulose, xylan has the highest extractability as compared with other polysaccharides, particularly with acid or alkaline treatment. During the delignification and hydrolysis processes, xylan would undergo the solubilization prior to the cellulose. The water solubilization of xylan compounds starts at 150 ^{o}C, which is also affected by the moisture content and pH (Fengel, 1984).

Although xylan has complicated structure, it also has the potential to be converted in the bioprocess due to its high pentose sugar content (~10–25% by dry

weight basis of the lignocellulosic biomass). However, the hydrolysate of xylan, e.g., xylose, is less preferable for the microorganisms rather than the hydrolysate of cellulose, glucose. This is due to the generation of adenosine triphosphate (ATP) from xylose is more complicated as compared with glucose, known as gluconeogenesis phenomenon (Janati-Idrissi et al., 1989). Thus, in most of biofuels production, the presence of xylan is considered as a hindrance and tends to be removed instead of utilized (Saha et al., 2005; Taherzadeh and Karimi, 2008). However, removal of xylan increases the mean pore size of the lignocellulosic biomass, thus increases the accessibility of enzymes for cellulose hydrolysis (Grethlein, 1985; Gregg and Saddler, 1996a; Palonen et al., 2004).

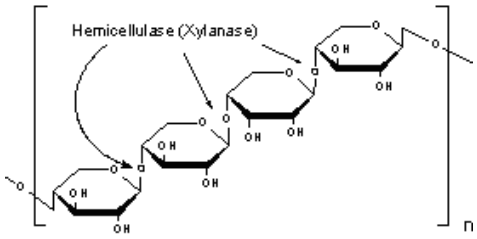


Fig. 2.5. Xylan chemical structure *(Hopkins, 1999)*

2.4.3. Lignin

Lignin is a complex molecule and considered as the most recalcitrant to the conversion. This is due to its water-insoluble amorphous heteropolymer structure (Fengel, 1984). It plays an important role in the plant cell wall to support the integrity, structural rigidity, and prevention of swelling of lignocellulosic biomass. Several phenylpropane units are linked in a three dimensional structure, which makes lignin or lignocellulosic biomass hardly to be utilized by microorganisms as growth substrate.

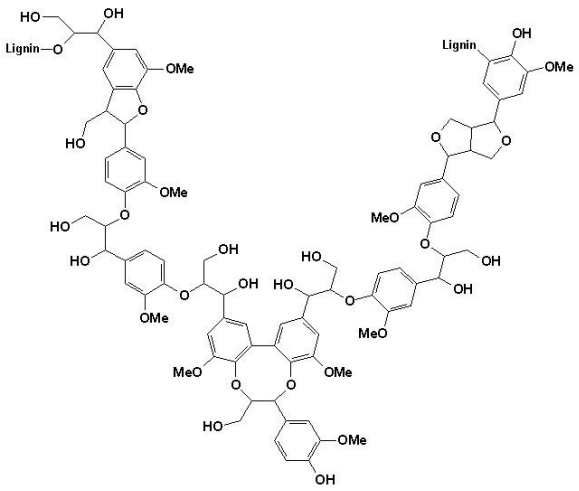


Fig. 2.6. Lignin chemical structure *(Hopkins, 1999)*

2.5. Energy extraction approaches from the lignocellulosic biomass

In order to extract the trapped energy of the lignocellulosic biomass, various processes can be performed, such as, (i) physical, chemical, or thermal process, and (ii) biological process. This first process can be performed through pyrolysis and gasification process with the production of syngas-pyrolysis oil-char or through incineration process with the production of heat (exothermic). Pyrolysis and gasification processes are aimed to produce gaseous fuels (syngas), liquefied fuels (pyrolysis oil/char), and solid fuels (char) through an incomplete oxidation process. Pyrolysis is a conversion process of biomass at 350–700 ^{o}C, in the absence of air or oxygen. Gasification is a conversion process of lignocellulosic biomass at 800–900 ^{o}C temperature, with an insufficient supply of air or oxygen. Incineration is a complete combustion process of biomass at 700–1,100 ^{o}C, in the presence of excess air or excess oxygen (Demirbas, 2001; Goyal et al., 2008). One shared drawback from these pyrolysis, gasification, or incineration processes is that the need of high energy input due to the influx of heating performance to increase the temperature of at the startup

of the process (Demirbas, 2001; Goyal et al., 2008). However, biological process overcomes this problem with its mild reaction conditions. Some of the energy-rich products generated from biological process are methane (from methanogenesis process) and acetone-butanol-ethanol-hydrogen (from anaerobic fermentation process). Methanogenesis process converts the organic waste to methane which is initiated by hydrolysis and acidogenesis stages (Daniels et al., 1984), while anaerobic fermentation process that employs culture like solventogenic *Clostridia* is capable of converting organic waste to solvents (Ma and Hanna, 1999).

One of the drawbacks of physical, chemical, or thermal method is the high production cost of generated fuels due to the significant amount of chemicals and energy addition in the process. This is further hindered with the non environmental friendly process as it generates chemical waste as by-products (Mosier et al., 2005; Taherzadeh and Karimi, 2008; Zhao et al., 2009). The energy production process should consume less amount of energy during the process, thus generates a net energy gaining. This has further aligned the search into biological process for the fuels production, which is considered to have milder condition due to the less energy and chemical consumptions during the process as compared to physical-chemical-thermal process. Furthermore, the process is considered more environmentally benign (Das and Veziroglu, 2001). Hence, the use of biological process that employs wild-type or natural microorganisms, such as bacteria and fungi, can further reduce the environmental impacts and ecological footprints (Magnani et al., 2007; Gomez et al., 2008).

2.6. Delignification and hydrolysis processes as a prerequisite for fermentation process

Cellulose and xylan have the highest potential of energy as the carbon source for the biological process. However, the complex structure of the latter is further encumbered by the presence of lignin, thus inhibits the microorganisms to access, utilize, and convert the polysaccharides (McMillan, 1994). Several preliminary steps are required to reveal the hidden energy potential, prior to the microbial conversion of polysaccharide through fermentation process. This must be performed through lignin removal or delignification process, expose the polysaccharides for the hydrolysis process, reduce the polysaccharides crystallinity through hydrolyzing the polysaccharides to monosaccharides, followed by the conversion of monosaccharides to fermentative products via fermentation process. In most literatures, "the pretreatment" term is to aimed to remove lignin and xylan, reduce cellulose crystallinity, and increase the porosity of the biomass (Sun and Cheng, 2002). However, it is more appropriate to distinguish between delignification and hydrolysis processes rather than pretreatment process. This is due to the nature of the processes that are different and every lignocellulosic biomass has specific degree of lignin or polysaccharides proportion. Delignification and hydrolysis processes can be performed through physical, chemical, thermal, and biological methods.

In the absence of lignin, for instance cellulose and xylan as the substrates, hydrolysis process can be directly applied without the delignification process. Lignin is insoluble in water, however, its solubility in water increases at the temperature ~180 ^{o}C under neutral pH conditions (Bobleter, 1994). Other than temperature and pH, lignin solubility also depends on lignin precursor presence, such as, p-coumaryl, coniferyl, or sinapyl alcohol of the lignin (Grabber, 2005). Nonetheless,

delignification process produces inhibitory compounds as by-products of the process, such as, furfural and HMF (hydroxymethylfurfural), for the following fermentation process (Berlin et al., 2006; Taherzadeh and Karimi, 2008). Thus, the removal of the inhibitory compounds is compulsory prior to the fermentation process, to provide a favorable environment for fermentative microorganisms. The most common process to remove these inhibitory compounds is through overliming process (Ranatunga et al., 2000).

In term of hydrolysis process, insoluble cellulose can be hydrolyzed to produce the six carbon monosaccharides, which are predominated by glucose, followed by trace amounts of galactose and mannose. Insoluble xylan can be hydrolyzed to produce the five-carbon monosaccharides, which are predominated by xylose and trace amounts of arabinose. Addition of exogenous hydrolytic enzymes such as cellulase for hydrolysis of cellulose and xylanase for hydrolysis of xylan are commonly performed as biological method (Sun and Cheng, 2002).

The competitiveness of energy production from lignocellulosic biomass would be achieved when a technically feasible and economically viable process can be implemented. Currently the delignification process is performed through physical, chemical, thermal, or the combination of methods. The high energy and chemical consumption during this process has pushed up the process cost (Sun and Cheng, 2002; Himmel et al., 2007; Kumar et al., 2009). Furthermore, there is a necessity to further treat the chemical by-products that are generated at the post process. Alternatively, enzymatic hydrolysis as a biological method is more preferable due to its milder environmental condition, thus consumes fewer amounts of energy and chemical by-products (Sun and Cheng, 2002). Nevertheless, the milder condition of biological method for hydrolysis process is still coupled with the costly enzymes, as

the hiccup from this method (Mielenz, 2001; Himmel et al., 2007; Zhang et al., 2007). Overall, delignification and hydrolysis processes are still the bottleneck for an economically affordable of lignocellulosic biomass conversion.

According to the abovementioned conditions for delignification and hydrolysis, some criterions must be met to achieve an effective and affordable process. From substrate perspective, some efforts must be performed through: (i) minimizing the cost of lignocellulosic biomass provision, (ii) minimizing the cost of lignocellulosic biomass size reduction. From process standpoint, some guidance to achieve the goal, such as, (i) avoiding an excessive harsh process to minimize the degradation of polysaccharides while maintaining the decrease of the polymerization degree, (ii) ensuring the more reduced crystalline region of cellulose to be exposed with the hydrolysis enzymes, (iii) reducing the chemical and energy costs, (iv) avoiding the formation inhibitory compounds for the fermentation process, and (v) subduing the waste residue generation (Taherzadeh and Karimi, 2008).

Comparison of various physical, chemical, thermal, and biological methods of delignification and hydrolysis processes is summarized in Table 2.6.

Table 2.6. Various methods of delignification and hydrolysis processes

Methods	Examples	Changes in the lignocellulosic biomass	Conditions
physical	**milling** ball milling two-roll milling hammer milling colloid milling vibro energy milling **irradiation** gamma ray irradiation electron beam irradiation microwave irradiation	increase the accessible of surface areas and pore size, decrease in cellulose crystallinity, decrease in degree of polymerization	energy demanding, low performance in delignification, unusual for industrial application, less chemical requirement
chemical	**explosion** steam explosion ammonia fiber explosion (AFEX) CO_2 explosion SO_2 explosion **alkali** sodium hydroxide ammonia ammonium sulfite **acid** sulphuric acid hydrochloric acid phosphoric acid **gas** chlorine dioxide nitrogen dioxide sulphur dioxide **oxidizing agents** hydrogen peroxide wet oxidation ozone **solvent extraction of lignin** ethanol - water extraction benzene - water extraction ethylene - glycol extraction butanol - water extraction swelling agents	increase the accessible of surface areas, partially or completely delignification, decrease in cellulose crystallinity, partially or completely hydrolyze xylan, decrease in degree of polymerization	the most effective in industrial application, high treatment rate, harsh environmental condition, high chemical requirement
thermal	**heating** hydrothermal high pressure steaming expansion extrusion pyrolysis	increase the accessible of surface areas, partially or completely delignification, decrease in cellulose crystallinity, partially or completely hydrolyze xylan, decrease in degree of polymerization	the most effective in industrial application, high treatment rate, harsh environmental condition, high chemical requirement
biological	**fungi and *Actinomycetes*** external enzyme addition microbes addition	delignification, decrease in degree of polymerization of cellulose, partially hydrolyze xylan	low energy requirement, no chemical requirement, mild environmental condition, low treatment rate, unusual for industrial application

Ref: (Taherzadeh and Karimi, 2008)

2.6.1. Physical method

Physical method is mostly aimed to reduce the particle size of the lignocellulosic biomass, thus increases the surface area and the accessibility of the enzymes to convert the polysaccharides (Taherzadeh and Karimi, 2008). The decrease of particle size and increase of available surface area would enhance the delignification and hydrolysis process, rather than crystallinity reduction (Hendriks and Zeeman, 2009). Particle size reduction through the milling increases the shearing of the lignocellulosic biomass by 5–25% that depends on the type of lignocellulosic biomass, milling method, and milling duration. This results to the reduction of hydrolysis time by 23–59% , thus increase the hydrolysis rate (Fengel, 1984; Hendriks and Zeeman, 2009).

An important feature from physical method compared with the chemical method is the absence of inhibitory compounds as the by-products. However, size reduction is coupled with the high energy requirement (Cowling and Kirk, 1976; Ramos, 2003), thus increases the process cost (Fan, 1987). The high performance of physical method is currently attractive for industries, despite of the high cost. This provides an avenue to achieve similar performance with physical method in lower cost.

2.6.2. Chemical and thermal methods

In the chemical or thermal method, lignocellulosic biomass is usually treated under high temperature and pressure (~150–180 ^{o}C and above 1 atm), with the presence of chemical agents. At this condition, xylan would be dissolved at the early stage, followed by the lignin (Bobleter, 1994; Garrote et al., 1999). During the heating process, some parts of xylan are hydrolyzed and converted to acids that catalyze the subsequent xylan hydrolysis. In comparison, chemical agents, such as acids or

alkalines, facilitate for the further reduction of substrate crystallinity (Gregg and Saddler, 1996a; Liu and Wyman, 2003; Zhu et al., 2004). Nevertheless, harsh conditions of chemical and thermal methods give a consequence of the formation toxic phenolic compounds for the following fermentation process (Gossett et al., 1982). Notably the chemical and thermal methods have proven to be used in many industrial applications, due to its high performance of delignification and reducing the degree of polymerization. However, the presence of chemical waste after the process makes the process less environmental friendly and unsustainable. A milder condition consumes less amount of energy, less chemical addition, less generated by-products as waste, while maintaining the performance and suppressing the processing cost, is considered as a more sustainable way of the future delignification and hydrolysis processes (Taherzadeh and Karimi, 2008).

2.6.3. Biological method

Biological method has showed a promising trend in the future of biofuels production. It can solve the shortcomings of the physical, chemical, or thermal methods, due to less chemical and energy consumptions that decrease the cost and relatively absence to the formation of harmful chemical by-products (Fengel, 1984). However, the performance is still hindered with its low performance, but reveals an avenue for further improvement. Thus, this enables an environmental friendly and sustainable method to be further up-graded in an application-scale.

Due to those abovementioned superiorities of biological process, more scientific attentions have recently been invested, but it is not commonly practiced yet in the industrial level due to its slow reaction rate. Microorganisms that are mostly used for biological method are bacteria and fungi (Duff and Murray, 1996; Taherzadeh and Karimi, 2008). Lignin has been reported to be degraded by several

fungi enzymes, such as lignin peroxidase, Mn-dependent peroxidase, and mono-phenol oxidase (Lee, 1997). The degradability of lignin by the lignin-degrading enzymes depends on the type of fungal, fungal cell growth condition, accessibility of lignin to the enzymes, and bioreactor configuration. Some genera of bacteria are also recognized for their ability to perform delignification and hydrolysis processes, in which predominated by the anaerobic thermophilic bacteria, such as *Anaerocellum thermophilum* DSM 6725, *Caldicellulosiruptor saccharolyticus* DSM 8903 (Yang et al., 2009). Nevertheless, thermophilic condition consumes higher amount of energy compared with mesophilic condition, in spite of thermophilic condition offers several process benefits, such as lower amount of energy for mixing due to the lower viscosity of fermentation broth and the ease of products recovery (Sun and Cheng, 2002).

2.6.3.1.Microorganisms

In term of microorganisms that can perform delignification and hydrolysis processes, fungi has shown its superiority compared with bacteria. Some fungi subdivisions, such as *Ascomycetes, Basidiomycetes*, and *Deuteromycetes* that have a diversity ~15,000 species for each, are the galore of the cellulolytic fungi (Carlile, 1997). Some of the genera commonly known as cellulolytic fungi are *Bulgaria, Chaetomium* and *Helotium* (*Ascomycetes* subdivision), *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (*Basidiomycetes* subdivision), *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium*, and *Trichoderma* (*Deuteromycetes* subdivision) (Carlile, 1997). The high rate of fungi growth coupled with the faster and abundant of cellulase production has attracted more studies of the cellulase production from fungi rather than bacteria (Lynd et al., 2002). Thus, the abundance of cellulase from aerobic microorganisms has surfaced

the search for cellulase from aerobic microorganisms other than fungi, such as bacteria.

For bacteria, there are several diverse physiological groups consisting of fermentative anaerobes that mostly are gram-positive bacteria (*Clostridium, Ruminococcus*, and *Caldicellulosiruptor*), gram-negative bacteria (*Butyrivibrio, Acetivibrio*, and *Fibrobacter*), aerobic gram-positive bacteria (*Cellulomonas* and *Thermobifida*), and aerobic gliding bacteria (*Cytophaga* and *Sporocytophaga*) (Hungate, 1966; Sanchez et al., 1990; Svetlichnyi et al., 1990; Khan et al., 1994; Lin et al., 1994; Rainey et al., 1994). There is a distinct hydrolysis mechanism between aerobes and anaerobes. Anaerobic bacteria utilize cellulose via complexed cellulase systems that attribute by polycellulosome organelles. Cellulase as the enzyme is excreted both extracellularly in liquid phase and on the surface of the cells, in trace quantity. Nevertheless, some species only own the localized complexed cellulases on the surface of the cells (Schwarz, 2001). In comparison, aerobic bacteria's cellulase system is relatively resembled with fungi's system. They utilize cellulose through the secretion of abundant extracellular cellulase as the predominant cellulase system, instead of the attached one at the cells surface (Bond and Stutzenberger, 1989; Wachinger et al., 1989; Schwarz et al., 2001).

Despite of the anaerobic system that employs low energy requirement process, the smaller quantity of excreted enzymes by anaerobes has overshadowed the search for enzymes production from anaerobes compared to aerobes (Wilson and Irwin, 1999). Therefore, the search for the novel anaerobic isolates or cultures that can perform cellulolytic and xylanolytic at the mesophilic condition would emerge as a breakthrough. Cellulolytic and xylanolytic properties might have been acquired by several bacteria. However, the fermentation performance of the isolates by directly

converting cellulose and xylan to biofuels would reveal an avenue for the biofuels production through a direct conversion process (Dumon et al., 2011; Suvorov et al., 2011; Yang et al., 2011).

2.6.3.2.Cellulase as the enzyme

Hydrolysis of cellulose is performed by cellulase, while hydrolysis of xylan is performed by xylanase. In order to hydrolyze and metabolize the insoluble cellulose, extracellular cellulase must be produced that are either free or cells-associated. Cellulase itself consists of a mixture of several enzymes (Doi et al., 1998). The major groups of cellulase that involved in the hydrolysis process include: (i) endoglucanase (endo-1,4-D-glucanohydrolase), that functions as free-chain ends creator in the low crystallinity region of the cellulose fiber, thus will open the structure into a free-chain ends. (ii) exoglucanase or cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase), that functions to degrade the molecule by removing cellobiose units from the free-chain ends. Cellobiose itself serves as the intermediate product of cellulose conversion into monosaccharides (Reese et al., 1959; Kabyemela et al., 1998). However, cellobiose is also a product inhibition as its inhibitory effect for the cellulase. This creates a condition that the produced cellobiose must be further converted into glucose, by using another compound inside the enzyme, β –glucosidase (Reese et al., 1959; Kabyemela et al., 1998). (iii) β –glucosidase, that functions to hydrolyze cellobiose to produce glucose. The produced glucose is also an inhibitory product for the β –glucosidase. The presence of excessive glucose will inhibit β –glucosidase performance in cellobiose conversion, thus the utilization of produced glucose for the downstream process, such as separation or further utilization through fermentation process must be performed to maintain the hydrolysis process performance (Lynd et al., 2002).

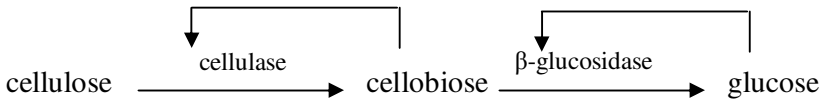


Fig. 2.7. Products inhibition of cellobiose and glucose in the hydrolysis process *(Doi et al., 1998)*

2.6.3.3. Cellulase attachment mechanism

Cellulase as modular structure consists of catalytic and carbohydrate-binding modules (CBMs). The CBMs facilitate the cellulose hydrolysis by attaching the catalytic domain to the substrate. Thus, the presence of CBMs is important for the initiation of the hydrolysis through exoglucanase exertion (Teeri et al., 1998). The proposed model of cellulose degradation (Reese et al., 1950) states the cellulase system is a multi effect of synergism, that encompasses (i) endo-exo synergy between endoglucanase and exoglucanase, (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (iii) synergy between exoglucanases and β-glucosidase to eliminate inhibitory cellobiose as an end product of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs.

In comparison for attachment mechanism of cellulolytic fungi and anaerobic bacteria, the fungi have the ability to penetrate cellulosic substrates through hyphal extensions. This affects to the closer distance between the excreted cellulase and cellulose, therefore an efficient hydrolysis can occur. This high-molecular cellulase weight and unstable enzyme system is termed as “noncomplexed” system (Lynd et al., 2002). In differentiation with fungi, anaerobic bacteria, such as *Clostridia*, do not have an ability to penetrate the cellulose side. The organelle that *Clostridia* own as its “complexed” systems or cellulosomes is through positioning the cellulase-producing cells at the site of hydrolysis (Lynd et al., 2002). When the enzymes seep to the lignocellulosic biomass, cellulase is commonly trapped and improperly functions

efficiently in the pores if the internal area is much larger than the external area (Zhang and Lynd, 2004). Lignin as the inhibitor for the hydrolysis process further shields the accessibility of enzymes to cellulose and xylan in lignocellulosic biomass (Chang and Holtzapple, 2000).

2.6.3.4.Cellulase loading

The higher ratio between the amount of cellulase and cellulose will increase the hydrolysis rate and subsequently the produced monosaccharides yield. However, the pricey cellulase commonly limits the loading of cellulase for biofuels production. The units of FPU and CBU usually determine the amounts of enzymes. FPU (filter paper unit) is the amount of glucose (in μmol) produced for every gram of cellulose per milliliter of enzyme per minute, while CBU (cellobiose unit) is the amount of pentose sugars (in μmol) produced for every gram of xylan per milliliter of enzyme per minute (Gregg and Saddler, 1996b). Cellulase loading is usually in the range of 7-33 FPU/g cellulose (Gregg and Saddler, 1996b), however, 10 FPU/g cellulose is the most commonly used in the enzymes study. This amount is considered cost affordable and reasonable hydrolysis performance that can be achieved within 48–72 hours of process (Esterbauer et al., 1991; Shaw et al., 2008). In comparison, the loading of β-glucosidase is generally practiced at the range of 2–3 CBU/g xylan (Gregg and Saddler, 1996a). Different types of lignocellulosic biomass need different amount of enzymes, due to the difference of cellulose-xylan-lignin configuration, crystalinity of the cellulose-xylan, and the degree of previous delignification process that has been performed on it (Gregg and Saddler, 1996b). Addition of β-glucosidase enhances the hydrolysis process performance, as it acts as cellobiose converter to monosaccharides. Currently, the most common commercial crude cellulase is extracted from fungi *Trichoderma reeseei*, which lacks of β-glucosidase content. Addition of the external

β-glucosidase would improve the achievement up to 90-95% of the conversion from cellulose into glucose and xylan to xylose, galactose, arabinose, and mannose. Fungi *Aspergillus niger* is the common source for crude β-glucosidase extraction (Excoffier et al., 1991).

Overall, a proper combination of delignification and hydrolysis processes is required. Generally, physical, chemical, or thermal method is applied for the delignification, followed by biological method for the hydrolysis process. However, the harsh condition in the delignification process becomes an obstacle due to the requirement of high amount of energy and chemicals, the production of chemical by-products, and high ecological or carbon footprint (Lynd et al., 2008; Pamela et al., 2009). This has hampered the process chain to be a sustainable and economically affordable process. A proposed process chain that can overcome the shortcomings of this unsustainable process chain is by combining all of the delignification, hydrolysis, and fermentation processes in a simpler process and single reactor, which is termed as consolidated bioprocess or CBP (Yang et al., 2009). Overall, the fermentation of lignocellulosic biomass is shown in Fig 2.8.

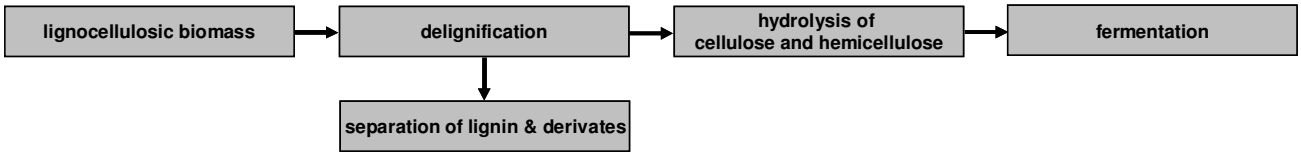


Fig 2.8. Process chain for fermentation process from lignocellulosic biomass

2.7 Fermentative butanol as an alternative fuel

Currently, one of the most promising liquid energy forms is butanol, beside ethanol. Actually, bioethanol production is still very common due to its most mature

bioprocess for fuel production, as it has been produced for centuries from grain crops. Furthermore, its production is also in line with the current needs of alternative fuels (Swan et al., 2011). Ethanol itself in USA has beeen produced at an annual capacity of 18.3 million m^3 in 2006, by using starch-rich corn kernels (Angenent, 2007) and is expected to be increased significantly up to 28.4 million m^3 in 2012 (Balat and Balat, 2009). South American countries including Brazil are still targetting to achieve 22% bioethanol usage in their vehicles fuel (Balat and Balat, 2009). The European Union sets a target of 5.75% contribution to overall consumed gasoline by 2010 (Balat and Balat, 2009). Overall, worldwide bioethanol production is expected to achieve 125 million m^3 by 2020 (Balat and Balat, 2009). However, there are several disadvantages of bioethanol compared with other alternative biofuel, e.g., the biobutanol, due to its chemical properties, as presented in Table 2.7.

Table 2.7. Chemical characteristic comparison between butanol and ethanol

Parameters	Butanol	Ethanol
energy density (i.e., gasoline = 34.2 MJ/L)	29.2 MJ/L	24.0 MJ/L
heat of vaporation	0.43 MJ/kg	0.92 MJ/kg
corrosivity	less corrosive	more corrosive
hydroscopicity	less hydroscopic	more hydroscopic
immiscibility with gasoline/diesel fuel	more miscible	less miscible
blending with gasoline/diesel fuel	8-16 %	5-10 %
engine modification	not necessary	necessary

Ref: (Ezeji et al., 2007b)

Butanol which is produced via biological route from lignocellulosic biomass is more preferable than the one from petrochemical route, due to its sustainability by using renewable resources as the substrate. Butanol is a colorless chemical, flammable liquid with a banana-like odor. It is miscible with all common solvents, but only sparingly soluble in water (Dürre et al., 2008). In term of health effect, it may

irritate the eyes and skin in a direct contact, may cause a narcotic effect if it is inhaled (Dürre, 2008). Other than the usage as fuel or fuel additive, butanol is also an important chemical for food processing, industrial solvents, paint, and varnish industries. Butanol also acts as a replacement chemical for anti-knocking in combustion engine, hydraulic and brake fluids, perfume production, safety glass, detergents, flotation aids, deicing fluid, cosmetics, antibiotics, camphor, hormones and vitamins (Dürre, 2008). In comparison with other common alternative fuels, ethanol, butanol shows its superiority in term of chemical properties as fuel, as presented in Table 2.7.

However, the cheaper production cost of butanol through petrochemical route, has retarded the butanol production from biological route. The low final concentration of butanol (~15-16 g/L) in the fermentation broth due to the toxicity of butanol to the microbes creates the high-energy demand for the downstream separation process (Groot et al., 1992; Ezeji et al., 2007b). This hiccup has shut down many biobutanol production plants worldwide since 1980s, when the fossil fuels price declined. Some of the big plants located in Peoria (the USA), Germiston (South Africa), and the former Soviet Union. The one in China was closed in 1990s (Chiao and Sun, 2007). The cheaper fossil fuels created a market condition to produce butanol through petrochemical route rather than biological route, which was considered cheaper (Gabriel 1928; Rose, 1961; Jones, 2001; Zverlov et al., 2006; Ni and Sun, 2009). Since then, butanol fermentation has been over-competed economically with the one that produced via chemical processes (Lee et al., 2008). However, the uncertainty of geopolitical condition in oil-producing countries in the recent years, followed by the more stringent environmental regulation, has revamped the outlook of fermentative butanol production. An improved butanol production in a more technically feasible

and economically viable process is necessary to overcome the past shortcomings of this biological production route (Jones and Woods, 1986; Lee et al., 2008).

Currently, the technological advancement has shown its higher avenue in term of industrial-scale application of butanol production. DuPont and UK based company British Petroleum (BP) recently set their intention to invest in biobutanol production in 2005. Some of their technological breakthroughs to achieve a feasible and affordable biobutanol production have been patented by using bio-based as the substrate (Qureshi et al., 2008; Kumar and Gayen, 2011). The operation of these companies has also been approved by European Commission, which was targeted to achieve an affordable butanol production at industrial-scale.

2.7.1. Substrates

Butanol is a fermentative product of monosaccharides via solventogenic fermentation pathway. As butanol fermentation pathway is initiated by Krebs cycle or tricarboxylic acid (TCA) cycle and glucose as the substrate (Lee et al., 2008), nonetheless the presence of monosaccharides is compulsory to initiate the process. If the substrates are polysaccharides, hydrolysis as the prerequisite process is necessary to be performed to decrease the polysaccharides crystallinity. The subsequent produced monosaccharides would be the readily-substrate for butanol fermentation (Lee et al., 2008). In the case of lignocellulosic biomass as the substrate, delignification process must be performed prior to the hydrolysis and butanol fermentation processes (Ezeji and Blaschek, 2008; Lee et al., 2008).

Biobutanol was originally produced as 1st generation of biofuels, as the substrate was originated from food source, such as corn liquor, potato starch, cassava starch, and molasses (Chiao and Sun, 2007). However, the food price hiked recently in 2006 to 2008 (Yang et al., 2009) has raised a concern for the sustainability of 1st generation of biofuels due to the competition for food. Alternatively, a cutting-edge approach was developed to produce 2nd generation of biofuels that use non-food source substrate, such as lignocellulosic biomass, due to its abundance and independency from the food supply chain. The fermentation route for polysaccaharides that contained in the lignocellulosic biomass is shown in Fig. 2.9.

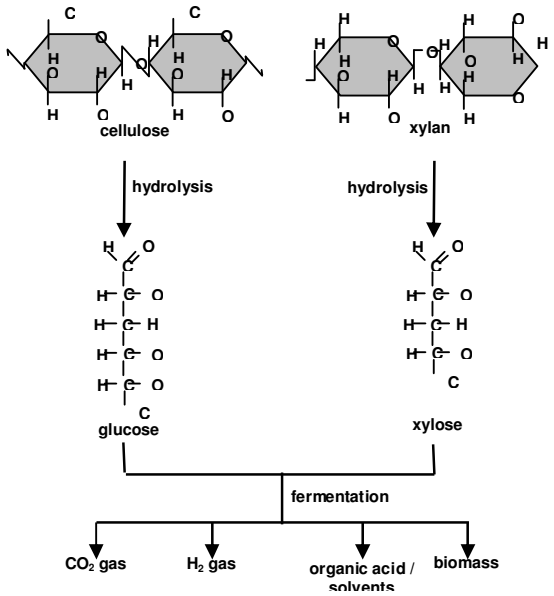


Fig. 2.9. Hydrolysis and fermentation routes *(Ezeji et al., 2007b)*

2.7.2. Microorganisms

Bacteria, specifically from *Clostridia* genus, perform most of the bioprocesses for butanol production. As butanol is produced together with acetone and ethanol as solvents, this fermentative pathway is usually termed as solventogenic pathway.

Among *Clostridia,* solventogenic bacteria are predominated by *C. acetobutylicum*, *C. beijerinckii*, and *C. pasteurianum* (Dürre, 2005). All of these *Clostridia* are mesophilic, rod-shaped, spore forming, gram positive, and strict anaerobic bacteria. These bacteria are able to utilize a wide range of five- and six-carbon monosaccharides, but mostly non-cellulolytic and non-xylanolytic (Lee et al., 2008). Some of the performance of wild-type solventogenic *Clostridia* is summarized in Table 2.8.

Table 2.8. Solventogenic performance of various *Clostridium* strains

Microorganisms	Fermentation time (hours)	Total produced solvents (g/L)	Monosaccharides preferences
C. acetobutylicum 260	60	20.3	glucose > cellobiose > mannose > arabinose > galactose > xylose
C. acetobutylicum 824	72	18.4	glucose > arabinose > xylose > cellobiose > galactose > mannose
C. saccharobutylicum 262	72	14.3	glucose > arabinose > galactose > cellobiose > xylose > mannose
C. butylicum NRRL 592	60	19.7	glucose > cellobiose > mannose > arabinose > galactose > xylose
C. beijerinckii BA101	72	18.0	cellobiose > glucose > xylose > arabinose > mannose > galactose

Ref: (Ezeji and Blaschek, 2008)

The efficacy of other solventogenic bacteria is also determined from the butanol yield, rate, productivity, and the type of utilizable substrates. *C. beijerinckii* has shown its superiority as its ability to utilize the hydrolysate from lignocellulosic biomass with remarkable performance (Qureshi and Blaschek, 1999; Qureshi and Blaschek, 2000a; Qureshi and Blaschek, 2000b). *C. aurantibutyricum*, another species of *Clostridia* showed a unique fermentation pathway by producing acetone and

isopropanol, instead of n-butanol, by using palm oil mill effluents as the substrate (Somrutai et al., 1996). Another improvement was performed by co-culturing several microorganisms, such as aerobic bacteria *Bacillus subtilis* TISTR 1032 with anaerobic bacteria *C. butylicum* WD 161. This co-culture resulted to 5.4-6.5 folds of solvents production compared to *C. butylicum* WD 161 alone as solventogenic bacteria, by using soluble cassava starch as substrate (Kumar and Gayen, 2011). The latter highly improved fermentation performance was caused by the amylase activity of the aerobic bacteria that enhanced the hydrolysis process of the substrate. This mutualism symbiosis resulted to the efficient utilization of liberated monosaccharides by the solventogenic anaerobic bacteria. Notably, this type of co-culture system favored in omitting the costly reducing agents, as which is usually required to maintain the growth of strict anaerobic *Clostridia* (Cheirsilp et al., 2010). However, the difficulty in avoiding culture contamination and infection, especially by the bacteriophage, has become the drawback of the co-culture system that employs aerobic process at the initial stage of the process (Cheirsilp et al., 2010). This shortcoming must be overcome through decontamination processes, such as sterilization, disinfection and immunization of resistant strains (Jones et al., 2000).

Several attempts to improve the performance of the solventogenic strains through metabolic engineering have shown a promising progress. There are some of the various successful genetic modifications in the previous studies. (i) Improve the acetone production from *C. acetobutylicum*. This was performed by amplifying of the acetone formation pathway, specifically formation of butyryl-CoA from butyric acid. This triggered the amplified adc (encoding acetoacetate decarboxylase) and ctfAB (encoding CoA transferase) genes. The acetone-forming enzymes became active earlier due to the amplified abovementioned genes, which lead to an earlier induction

of acetone formation. Overall, final concentrations of acetone, butanol, and ethanol improved by 95%, 37%, and 90%, respectively, compared to its parental strain (Mermelstein et al., 1993). (ii) Overexpress the AdhE gene that shifts the fermentation pathway to produce more ethanol than butanol, in comparison with the parental strain. (Nair et al., 1994). (iii) Induce Spo0A gene during the acidogenic stage and suppress it during the solventogenic stage, in which a putative regulator of the sol operon in *C. acetobutylicum*. This was coupled with the removal of solR gene region to enhance butanol production (Scotcher and Bennett, 2005). (iv) Inactivate buk and pta genes that encode butyrate kinase and phosphotransacetylase. This was aimed to redirect the carbon flow from acidogenic stage to solventogenic stage. This was performed in *C. acetobutylicum* PJC4BK, resulted with the 10% improvement of produced butanol compared with the parental strain (Harris et al., 2000). (v) Fine-tune gene expression by transforming bukasRNA gene that resulted to the improved final concentration of acetone and butanol by 50% and 35% compared with the parental strain. This also resulted with the decrease of butyric acid production as the by-product of solventogenic fermentation up to 65–75% (Desai and Papoutsakis, 1999). (vi) Utilize genomic library for simplifying the screening and genes characterization that were capable to improve butanol tolerance (Borden and Papoutsakis, 2007). Overall, metabolic engineering can be performed in order to rectify the genes that are responsible for butanol production, hence de-activate the genes that responsible for by-products formation. This influences to the more efficient fermentation pathway, as more carbon is directed to solvents, rather than non-solvents products (Lee et al., 2008). The culture's ability to utilize wider range of substrates and withstand high concentration of butanol can also be modified at the genetic level. This would enable

more various types of substrate candidates for butanol production, including lignocellulosic biomass (Lee et al., 2008).

2.7.3. Growth conditions

All solventogenic bacteria are strict anaerobic bacteria (Dürre, 2005). High reduction oxidation potential is required to ensure high butanol and ethanol productions that compensated with the reducing acetone production (Ezeji et al., 2004). Addition of a pricey complex nitrogen sources, such as yeast extract-peptone (YEP), is commonly practiced in solventogenic fermentation (Ezeji et al., 2004; Ezeji and Qureshi et al., 2007; Ezeji and Blaschek, 2008). Both performs in solventogenic fermentative pathway by converting monosaccharides to acetic acid, butyric acid, acetone, butanol, ethanol, carbon dioxide, hydrogen, and cells (Dürre, 2005).

Biobutanol production process is initiated by the acidogenic stage, in which acetic acid, butyric acid, hydrogen, and carbon dioxide are generated, coupled with the exponential growth of cells. This stage decreases the pH from neutral to ~4.0–5.0 (Lee et al., 2008) that creates an unfavorable environment for the cells due to the acidic pH. This unfavorable condition forces the cells to shift to sporulation stage as the self-defense mechanism (Lee et al., 2008). When the external pH drops to less than 4.5, undissociated acids diffuse into the cytoplasm that increases the external pH (pHe). This pH difference causes pressure on the membrane due to the proton gradient, which results to the breakdown of cells (Dürre, 2008). Solventogenic *Clostridia* as acidogenic bacteria can survive at low pH by re-assimilating the produced acetic acid and butyric acid, to neutral compounds, such as acetone, butanol, and ethanol as solvents. The conversion of acids to solvents raises the pH and coupled by the produced solvents, but decoupled with the cell growth. This is a distinct property of solventogenic bacteria in term of its survivability to the acids presence, in

which conversion of acids to solvents serves as surviving strategy (Lee et al., 2008). Indirectly, the low pH is a prerequisite to induce solvents production (Kim et al., 1984), however, extreme low pH (~< 4.0) tends to lyses the cells. pH of the fermentation system is usually buffered to increase the cell growth, high substrate utilization, and high butanol production (Bryant and Blaschek, 1988), concurrently to avoid the cells lyses. Apart from the produced solvents, biofuel in the gaseous form is also produced, such as hydrogen, coupled with the presence of carbon dioxide. Proportion of hydrogen in the biogas mixture changes accordingly, which decreases within the fermentation time with the rise up of carbon dioxide proportion (Fouad et al., 1976; Ezeji et al., 2007b; Dürre, 2008; Lee et al., 2008). Different cell stages in solventogenic fermentation are showed in Fig. 2.10.

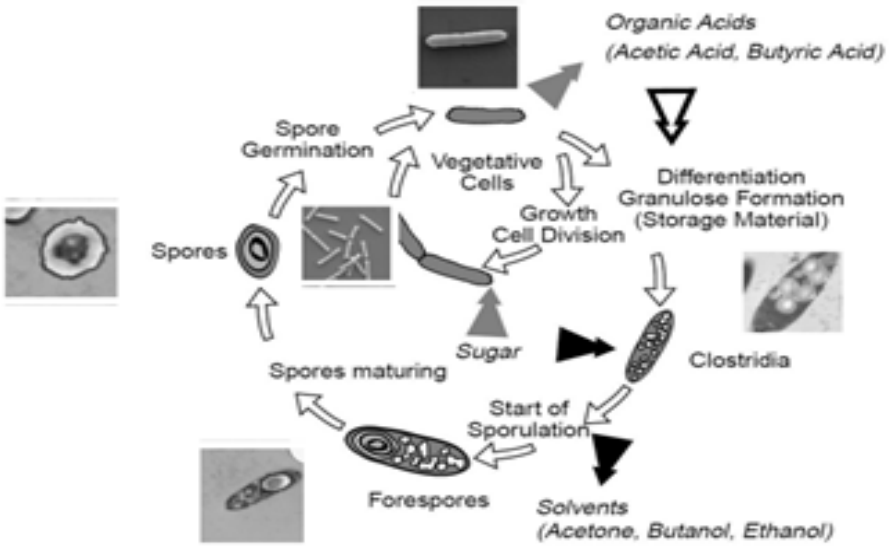


Fig. 2.10. Cell stages in solventogenic fermentation *(Schuster et al., 1998)*

Fermentation process takes ~7 days until it comes to the stationary and death phases. The stationary phase and death phases start to be onset as the depletion of substrate and/or the presence of toxic butanol. Depletion of the substrate will cease

the cells growth, thus the death stage starts (Lee et al., 2008). Butanol as one of the solventogenic products is the most toxic product compared with acetone and ethanol. This is due to the lipophilicity and toxicity of butanol compared with other solvents that increases the membrane fluidity, thus disrupts the phospholipid components at the cell membrane (Bowles and Ellefson, 1985). The increase of membrane fluidity causes destabilization of the membrane and disturbance to the membrane-associated functions such as transport processes, glucose uptake, and membrane-bound ATPase activity (Bowles and Ellefson, 1985). From this fact, butanol toxicity is one of the bottlenecks for a higher achievable butanol production. The presence of 7.4–11.1 g/L of butanol showed an inhibition effect to the cells growth (Moreira et al., 1981; Woods, 1995). Solventogenic fermentation pathway is shown in Fig. 2.11.

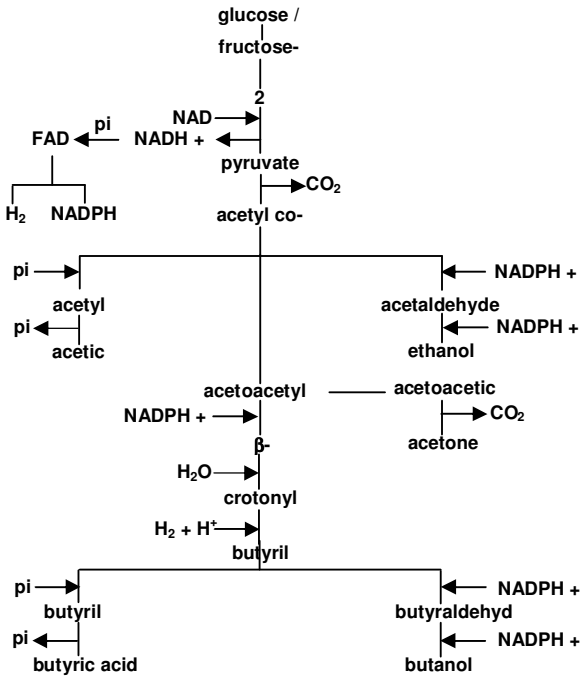


Fig. 2.11. Solventogenic fermentation pathway *(Lee et al., 2008)*

2.8. Direct conversion process

Direct conversion of lignocellulosic biomass to biofuels, as the combination of delignification, hydrolysis, and fermentation processes is considered as the cutting-edge of biofuels production. Many literatures classify this combined process as consolidated bioprocess, in short CBP (Lynd et al., 2002; Yang et al., 2009). However, the conversion of pure cellulose and xylan to biofuels may not be suitable to be termed as CBP, due to the absence of lignin content. This consideration rectifies CBP term to "direct conversion", which is more suitable.

Through combination of delignification, hydrolysis, and fermentation processes, an achievement of a more efficient, effective, economically viable, environmentally benign, and more sustainable process is expected. This direct conversion process is performed by employing microorganisms in pure culture or consortia, to break down the lignin, excrete out the hydrolytic enzymes, and simultaneously ferment the hydrolysate to biofuels (Lynd et al., 2005). Therefore, direct conversion as a combined process has to customize the different conditions between the processes. The different of conditions has contravened the overall performance of the process (Lynd et al., 2005), however, some studies have showed distinctive performances. Most of the recent studies (Svetlichnyi et al., 1990; Mladenovska et al., 1995; Lynd et al., 2005; Kataeva et al., 2009) showed the production of ethanol and hydrogen under thermophilic conditions. Nevertheless, the observed final concentration, rate, yield, and productivity were still considered low. Some of the microorganisms that enable direct conversion process are *C. thermocellum* and *Anaerocellum thermophilum* DSM6725 for lignocellulosic biomass (Lynd et al., 2005; Kataeva et al., 2009) . *Anaerocellum thermophilum* Z1320 and

Caldicellulosiruptor saccharolyticus DSM8903 were able to directly convert cellulose and xylan into ethanol, lactic acid, acetic acid, hydrogen, and carbon dioxide (Svetlichnyi et al., 1990; Mladenovska et al., 1995). The microbial performance under mesophilic conditions may be lower than that of thermophilic direct conversion, so certain mesophiles did fare better than thermophiles. For instance, a mesophile *Enterococcus gallinarum* G1 was capable of producing twice the amount of hydrogen from cellulose as compared to other thermophilic bacteria (Lo et al., 2008; Wang et al., 2009).

Some studies about direct conversion of polysaccharides or lignocellulosic biomass to ethanol production have been reported as abovementioned (Svetlichnyi et al., 1990; Mladenovska et al., 1995). However, there are no reports of the direct conversions to produce significant amounts of butanol. In a recent study (Higashide et al., 2011), a genetically modified *C. cellulolyticum* showed an ability to produce isobutanol directly from cellulose at mesophlic temperature. This was achieved by expressing the enzymes that convert pyruvate to isobutanol by using an engineered valine biosynthesis pathway, which resulted to the production of 660 mg/L of isobutanol. However, this amount of isobutanol was considered low and still uneconomically favorable for the downstream separation process, as compared with conventional butanol production that can reach ~15 g/L of butanol. As there are currently no reported cultures harboring such capabilities, there is a need for the discovery of pure cultures or consortia that can perform direct conversion of lignocellulosic biomass to butanol. It is hypothesized genetic manipulation from cellulolytic, xylanolytic, or solventogenic bacteria would enable the direct conversion of lignocellulosic biomass to butanol. Some of the previous successful genetically modified microorganisms were *Escherichia coli* (Srivastava et al., 1995; Yoo et al.,

2004), *Zymomonas mobilis* (Bresticgoachet et al., 1989; Moniruzzaman et al., 1997), and *Saccharomyces cerevisiae* (Tsai et al., 2009; la Grange et al., 2010), which are mainly for ethanol production.

2.9. Reactor operation and downstream separation process

Batch mode was initially considered as the most suitable operation mode for butanol production in the industrial-scale. This was considered upon its simple operation and the minimum risk of contamination. However, batch mode was not efficient due to butanol toxicity inhibition and longer down time for cleaning-sterilizing-filling activities (Ezeji et al., 2004b). In order to cope with these shortcomings, a modified continuous mode system was operated, in line with the real time separation for the produced butanol. Beside the real time separation, the amount of cells in the system was also kept high by applying an attached biological growth and cell recycle (Ezeji et al., 2004b).

Immobilized cell and membrane cell recycle are the most common reactor operation modes to improve the cell density for butanol production (Qureshi and Blaschek, 2000b). Culture can be grown and attached onto supportive biomass to support its growth as culture immobilization mode. As an example, *C. beijerinckii* BA101 was immobilized onto clay brick during the solventogenic fermentation. At a dilution rate of 2/h, 7.9 g/L, solvents were produced with a productivity of 15.8 g/L/h and a yield of 0.38 g/g. Thus, an immobilized cell fermentation in a continuous reactor with simultaneous solvents separation would be a good candidate for industrial operation mode (Qureshi and Blaschek, 2000b). A continuous cell recycle-equipped with membrane reactor employs a hollow-fiber ultrafilter to separate and recycle cells. A previous study showed the operation of this membrane at a dilution rate of 0.5/h that yielded 13 g/L of produced solvents by employing 20 g/L of *C.*

acetobutylicum cell. One of the drawbacks of this method was the membrane fouling that generated more downtime for the membrane operation due to the membrane backflush and cleaning (Lipnizki et al., 2000).

For the downstream separation of butanol, several modes have been developed. (i) Conventional distillation. This is a conventional mode for solvents separation. However, the low concentration of produced total solvents in the fermentation broth (< 20 g/L) makes this method not economically affordable (Dürre, 1998). (ii) Reverse osmosis. This mode can selectively separate the solvents, although the high operation cost and membrane clogging are always observed for its application (Dürre, 1998). (iii) Liquid–liquid extraction. This mode embarks a high capacity and selectivity for the separation in low energy requirement system. The phenomenon of distribution coefficients differences is applied in this method, in which butanol as a more soluble in the extractant (organic phase) to be separated from the fermentation broth (aqueous phase). However, the toxicity of the extractant to the cell often hinders the application of this mode (Dürre, 1998). (iv) Gas stripping. As a simple and efficient mode to separate butanol from the fermentation broth, an operation with high concentration of substrate, high substrate utilization, and minimum inhibition due to butanol can be achieved. The fermentation gas is bubbled through the fermentation broth prior to the recovery in a condenser. The stripped gas is then recycled back to the reactor and the process continues until all the monosaccharides deplete (Qureshi and Blaschek, 2001). (v) Pervaporation. A selective separation of volatile compounds from the fermentation broth is applied in this mode. Membrane is in contact with the fermentation broth and the volatile liquids or solvents as vapor will be diffused through the membrane. The separated product is then further recovered by condensation (Qureshi and Blaschek, 2000a).

Summary

This chapter has given an overview for the conversion of lignocellulosic biomass or polysaccharides to biofuels. Butanol that is produced via biological route is considered as one of the most promising biofuels. Currently, most of the known-strains for butanol production are not known to have high performance of cellulolytic and xylanolytic properties. This fact has pushed more studies for the separate delignification and enzymatic hydrolysis (Gutierrez et al., 1998; Ezeji et al., 2007a; Ezeji and Blaschek, 2008), instead of the direct conversion. However, the research hiatus for the absence of pure cultures or consortia that can produce butanol directly from lignocellulosic biomass must be filled. An initiation study to achieve the abovementioned gap is through an isolation of bacteria that harbor cellulolytic and xylanolytic properties. Microbe that can convert hydrolysate of polysaccharides, such as glucose and xylose to butanol, should be discovered. Co-culture of these isolates or pure cultures that can perform a simultaneous cellulolytic, xylanolytic, and solventogenic activity would be an important and promising milestone to be achieved. This would reveal a step forward of direct conversion as a cutting-edge process for biofuels production in the near future.

In terms of produced butanol, the low final concentration of butanol that can be achieved (< 16 g/L) must be overcome, in order to attain an efficient separation at the downstream process. This can be achieved by simultaneously operating a continuous reactor that employs an improved strain that has high resistance to the toxic butanol as product inhibition and populate at high cells concentration in the reactor. Thus, combination of a stable reactor system that accomplishes gradual shift from acidogenic to solventogenic stage and coupled with the simultaneous removal of produced butanol would further improve the butanol fermentation performance.

CHAPTER 3

A mesophilic *Clostridium* species that produces butanol from monosaccharides and hydrogen from polysaccharides

Abstract

A unique mesophilic *Clostridium* species strain BOH3 is obtained in this study, which is capable of fermenting monosaccharides to produce butanol and hydrolyzing polysaccharides to produce hydrogen (H_2) and volatile fatty acids (VFAs). From 30 g/L of glucose and xylose each, batch culture BOH3 was able to produce 4.67 and 4.63 g/L of butanol. Enhancement treatments by increasing the inoculated cells improved butanol production to 7.05 and 7.41 g/L, respectively. Hydrogen production (2.47 and 1.93 mmol) was observed when cellulose and xylan (10 g/L each) were used, suggesting that strain BOH3 possesses xylanolytic and cellulolytic capabilities. These unique features reveal the strain's novelty as most wild-type solventogenic strains have not been reported to have such properties. Therefore, culture BOH3 is promising in generating butanol and hydrogen from renewable feedstock.

Keywords: Butanol; Hydrogen; *Clostridium*; Cellulose; Xylan

3.1. Introduction

The search for sustainable energy calls for the usage of renewable resources as the feedstock. Apart from alleviating pollution problems, alcohols (e.g., butanol and

ethanol) converted from the cellulosic biomass are promising alternative fuels and can be used to fulfill the increasing energy demand (Lee et al., 2008; Atiyeh et al., 2011). Between butanol and ethanol, butanol is a choice of fuel as compared to ethanol, mainly because of its higher energy density, lower volatility and reduced corrosiveness. Additionally, butanol has relatively better compatibility for current car engines and infrastructures, offering more convenience and versatility in applications (Dürre, 2008; Swana et al., 2011). Thus, butanol generation from lignocellulosic materials has attracted much attention from contemporary researchers in the discipline of bioenergy.

Clostridia species (e.g., *Clostridium acetobutylicum*, *C. beijerinckii,* and *C. pasteurianum*) are known to be solventogenic in producing acetone, butanol, and ethanol, but are still subjected to negative inhibition by their own products (Ahn et al., 2011; Lee et al., 2008). Among all wild-type *Clostridia* bacteria, the highest reported butanol concentration was 11.7 g/L from the fermentation of glucose (Lee et al., 2008). The fermentation efficacy was reported to be hampered due to the accumulated butanol (e.g. >7.4 g/L) (Lee et al., 2008), which could lead to cell growth inhibition and premature cessation of fermentation (Ezeji et al., 2007). Such negative inhibition leads to low achievable butanol concentration and will thus increase the downstream costs associated with product purification (Ezeji et al., 2007). Attempts have been made to improve the butanol concentration up to 17.8 g/L by genetically manipulating the wild-type *Clostridia* species (Lee et al., 2008). Nevertheless, genetically modified bacteria are usually unstable due to plasmid excision (Heap et al., 2007), leading to the deterioration of butanol-producing capability within batches of experiments. Hence, the search for a novel and enhanced

wild-type microbes with improved butanol resistance is of great necessity for industrial applications.

Producing bioenergy from lignocellulosic biomass has been reported by several studies, but often was operated at harsh and energy-demanding conditions. For example, bacteria from genus of *Clostridia, Thermotoga*, and *Enterococcus* are reported to produce hydrogen, methane (Liu et al., 2008; Lo et al., 2008; Nguyen et al., 2008; Wang et al., 2009), and ethanol from cellulose (Sato et al., 1992) under thermophilic conditions. Some *Thermoanaerobacter* species have also shown capabilities of producing ethanol directly from xylan (i.e. typically constitutes 8-23% dry mass of wood), nevertheless only at elevated temperatures (70°C) (Ahring et al., 1996). Exceptionally, a *Clostridium pasteurianum* is capable of producing hydrogen directly from cellulose at mesophilic conditions, the amount of which is comparable with other thermophilic strains (Lo et al., 2008; Yang et al., 2009). Additionally, a *Clostridium* strain (Montoya et al., 2000) shows butanol and acetone generation from fermenting glucose. Though the culture also possesses cellulase and xylanase, its performance in the direct biofuels production from cellulose and xylan is not substantiated in the above study. Thus far, few studies have been successful in directly converting xylan and cellulose to biofuels without prior enzymatic hydrolysis (Pitkanen et al., 2005), while the conventional butanol generation from lignocellulosic biomass is via serial physical and chemical pretreatments (i.e., delignification process), enzymatic hydrolysis of polysaccharides into monosaccharides, and subsequent fermentation of monosaccharides to biosolvents (Qureshi et al., 2005; Ezeji et al., 2007; Ezeji and Blaschek, 2008). The drawbacks of the above processes are that the pretreatment stage is energy-consuming and may cause the formation of inhibitory compounds for the downstream fermentation process, and the commercial

enzymes (e.g., cellulases) used for hydrolysis are costly (Ezeji et al., 2007). Screening for a mesophilic microbe which possesses potential activity in cellulolytic, xylanolytic, and solventogenic is thus crucial to the bioenergy industry.

The aim of this study is to cultivate and characterize butanol-generating microbes via utilizing glucose and xylose at mesophilic conditions. The identified wild-type microbe – designated *Clostridium* species strain BOH3 is also investigated for its fermentation profile in response to cellulose and xylan to uncover the potential application of the culture in biofuels production from lignocellulosic feedstock.

3.2. Materials and Methods

3.2.1. Culture cultivation and characterization

A fully submerged soil sample from a paddy field was collected and inoculated into a N_2-purged, autoclaved bottle containing 10-mL of reinforced clostridial media (RCM Oxoid, UK). After 48-h incubation at 35°C and 125-rpm shaking speed, the culture was spread onto a petri dish containing Reinforced Clostridial Agar (RCA, Oxoid, UK) in an anaerobic chamber. A total of 50 colonies were randomly picked using sterilized inoculation loops to further streak on fresh petri dishes containing RCA. The individual colonies were then transferred to RCM liquid media under anaerobic conditions to test their butanol-generation capability. Subsequently, a butanol-generating culture named BOH3 was obtained by culturing cells from a single colony in a sterile RCM medium.

The genomic DNA of cell pellets from culture BOH3 was extracted and purified with DNeasy Tissue Kit (Qiagen GmbH, Germany). The 16S rRNA gene was amplified by PCR (Eppendorf, Germany) using a pair of universal primers 8F (5'-AGA GTT TGA TCC TGG-3') and 1392R (5'-CTC AGACG GGC GGT GTG T-3')

(Maniatis, 1982). The resultant product was concentrated and purified with PCR purification kit (Qiagen GmbH, Germany). Subsequently, the 16S rRNA was sequenced with the ABI DNA Sequencer. The sequence queries were then compared to the 16S rRNA gene sequence database through Basic Local Alignment Search Tool (BLAST). The nucleotide sequence of culture BOH3 was deposited in the GenBank database with an accession number HQ830243. Active culture BOH3 was observed periodically under light microscope, Nikon Eclipse E200 (Nikon, Melville, N.Y., U.S.A.). DNA of the culture was stained using fluorescence 4'-6-diamidino-2-phenylin-dole (DAPI) for microscope observation.

3.2.2. Growth conditions of culture BOH3

A reduced mineral salts medium (36-mL) was prepared (He et al., 2003) in a 60-mL serum bottle and supplemented with Wolin solution (Wolin et al., 1963). Glucose (30 g/L), xylose (30 g/L), microcrystalline cellulose (10 g/L), or xylan from oat spelts (10 g/L) were added individually as a carbon source. The pH of the defined medium was adjusted through the addition of 10% H_2SO_4 under continuous N_2 purging at the start of each experiment. The initial pH of cellulose- and xylan-containing cultures we controlled within a range of 7.0-7.2 while that of glucose- and xylose-containing cultures was between 5.0 and 5.5. Active culture BOH3 (10% [v/v], that is, 4-mL active culture to 36-mL fresh medium) was then inoculated into triplicate bottles for each substrate, and incubated at 35°C with a 125-rpm shaking speed.

The addition of butyric acid (0.7 g/L) and heat treatment of cell pellets (at 70ºC for 5 minutes) were conducted to enhance the performance of culture BOH3, while keeping same inoculation size (10% [v/v]) as above. Apart from these stresses, augmented inoculation was also applied to enhance the butanol concentration and

yield, by doubling the initial amount of inoculation cells. Prior to the inoculation, 8-mL of active culture BOH3 was centrifuged at 10,000 rpm for 10 minutes to double the cells. The supernatant was carefully decanted in the anaerobic chamber, followed by the addition of 4-mL sterilized mineral salts medium to re-dissolve the concentrated cells pellet. This 4-mL mixture was subsequently inoculated into a new bottle containing 36-mL medium. Apart from increasing the initial amount of cells, the augmented inoculation also minimized the effects of residual monosaccharides and volatile fatty acids (VFAs) from the previous culture.

Additionally, the tolerance of culture BOH3 on butanol was conducted via the initial addition of 5 or 10 g/L of butanol to the cultures containing glucose or xylose with the aforementioned augmented inoculation. Butanol tolerance was then assessed by comparing the final generated butanol and cell growths measured by using cellular protein amount with the controls (i.e., no initial butanol addition).

3.2.3. Chemical analysis

Samples were extracted from separate batch cultures for the identification and quantification of final products. The VFAs (i.e., acetic and butyric acids) and biosolvents (i.e., acetone, ethanol and butanol) were separated through gas chromatography (GC, model 7890A; Agilent Technologies, U.S.A.) on a Durabond (DB)-WAXetr column (30 m × 0.25 mm × 0.25 μm, model 123-7334; J&W, U.S.A.) and were measured with a flame ionization detector (FID), in which: the oven temperature was initially held at 60°C for 2 min, increased at 15°C/min to 230°C, and held for 1.7 min. Helium was used as the carrier gas, with a column flow of 1.5 mL/min. Five-point standard curves were obtained by running standard solutions containing acetone, butanol, ethanol, acetic acid, and butyric acid. The compositions of the gaseous products were determined with another GC (model GC-17A;

Shimadzu, Japan) equipped with a thermal conductivity detector (TCD) and a Supelco custom column 80/100 Porapak N column (2 m × 1/8 inch S.S). The oven holding temperature was kept constant at 110°C for 2.3 min and argon (15 mL/min) was used as the carrier gas. Standard gaseous mixtures consisting of hydrogen, nitrogen, carbon dioxide, and methane at known proportions were used to obtain the calibration curve.

The residual glucose or xylose concentrations in the samples were determined by using a high-performance liquid chromatography equipped with a refractive index detector (HPLC-RID) (model 10A; Shimadzu, Japan). An Aminex HPX-87P (300 mm × 7.8 mm) column was applied to separate glucose and xylose, using degassed millipore water (0.6 mL/min) as the mobile phase and constant column temperature at 70°C. The carbon mass balance was then calculated based on the concentrations of carbon element in both liquid and gaseous fermentation products. Cellular protein concentration was determined by using the Ettan 2-D Quant Kit (GE, UK) after cell lysis by a sonicator (Sonics Vibra Cell,model CV19; Sonics, U.S.A.) (Guerlava et al., 1998). The pH was measured by mini pH meter ISFET 120 and adjusted using sterilized 10% H_2SO_4 or 10% NaOH in the biosafety cabinet.

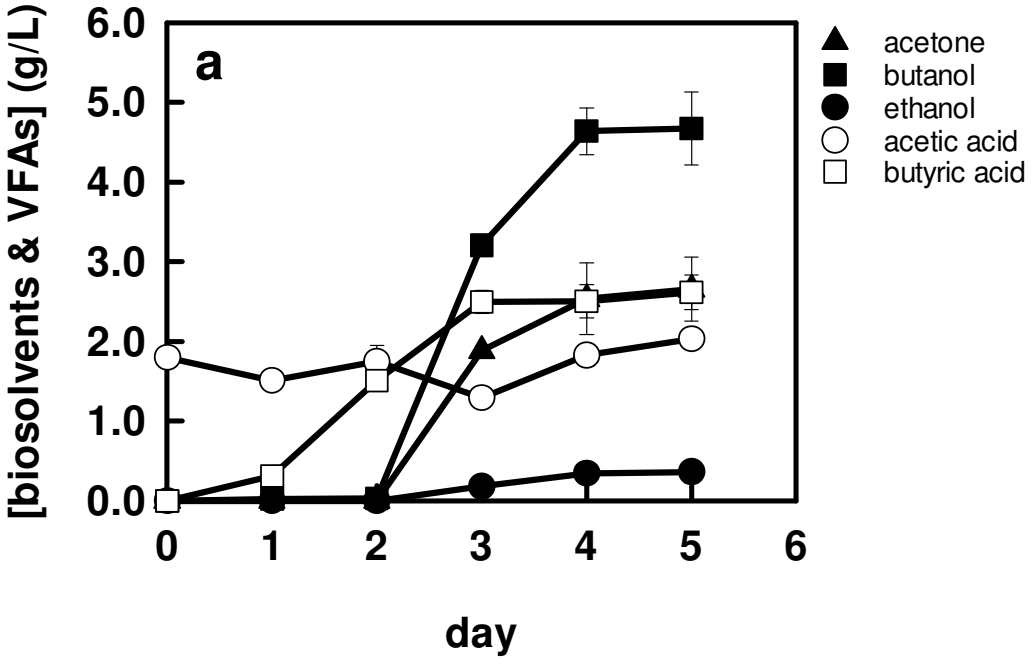
3.3. Results

3.3.1. Butanol production from glucose and xylose by culture BOH3 and its phylogeny

Culture BOH3 was obtained from an individual colony on a RCA petri dish, which shown butanol production when transferred to a mineral salts medium spiked

with glucose (30 g/L). After more than 5 transfers, this culture was capable of producing acetone, butanol, and ethanol at concentrations of 2.65, 4.67, and 0.36 g/L within 5 days of incubation (Fig. 3.1). Moreover, culture BOH3 could utilize xylose (30 g/L) to produce 2.88 g/L of acetone, 4.63 g/L of butanol, and 0.61 g/L of ethanol within 11 days of incubation (Fig. 3.2). At the same time, hydrogen of 5.40 and 4.22 mmol were also generated from the above glucose and xylose fermentation processes, and the proportion of hydrogen in the produced gas mixture was ~20 to 40%.

Culture BOH3's purity was confirmed by a clone library established with the 16S rRNA genes via yielding identical restriction patterns for the 70 clones after digesting with three enzymes, *Hha*I, *Rsa*I and *Msp*I. The sequence of the 16S rRNA genes amplified from the genomic DNA of culture BOH3 exhibited 98% identity to the sequence of *Clostridium butyricum* strain W4. Thus, culture BOH3 is designated as *Clostridium* species strain BOH3. *C. butyricum* was reported to be a group of endospore forming, motile, rod-shaped, obligate anaerobic prokaryotes that produce acetic and butyric acids, while its solventogenic properties has not been clearly expounded (Demattos et al., 1994; Montoya et al., 2001). Microscopic picture of culture BOH3 is showed in Fig 3.3.



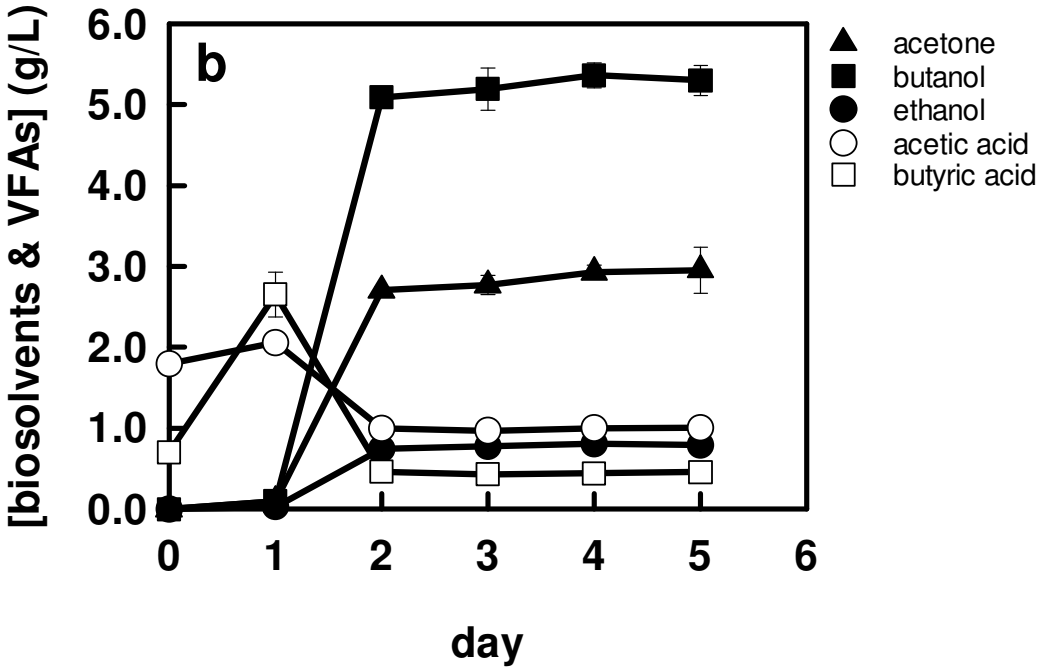
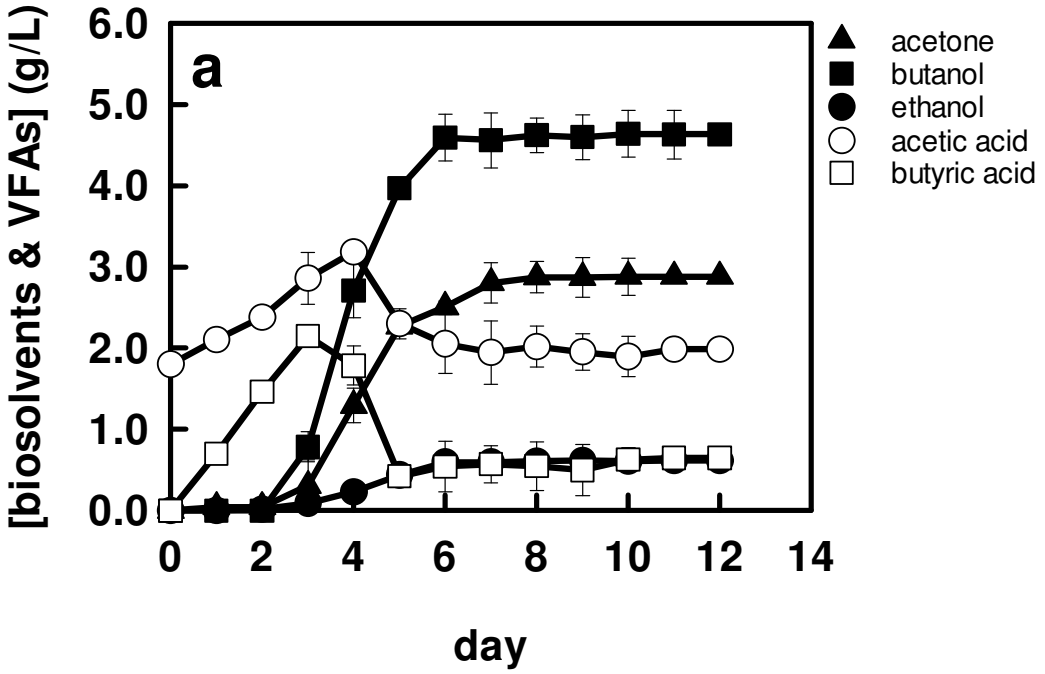


Figure 3.1. Biosolvents and VFAs production by *Clostridium* species strain BOH3 (10% [v/v] inoculation) with glucose as a substrate. (a) Fed with 30g/L of glucose solely; (b) Fed with 30g/L of glucose and 0.7 g/L of butyrate for butanol enhancement treatment.



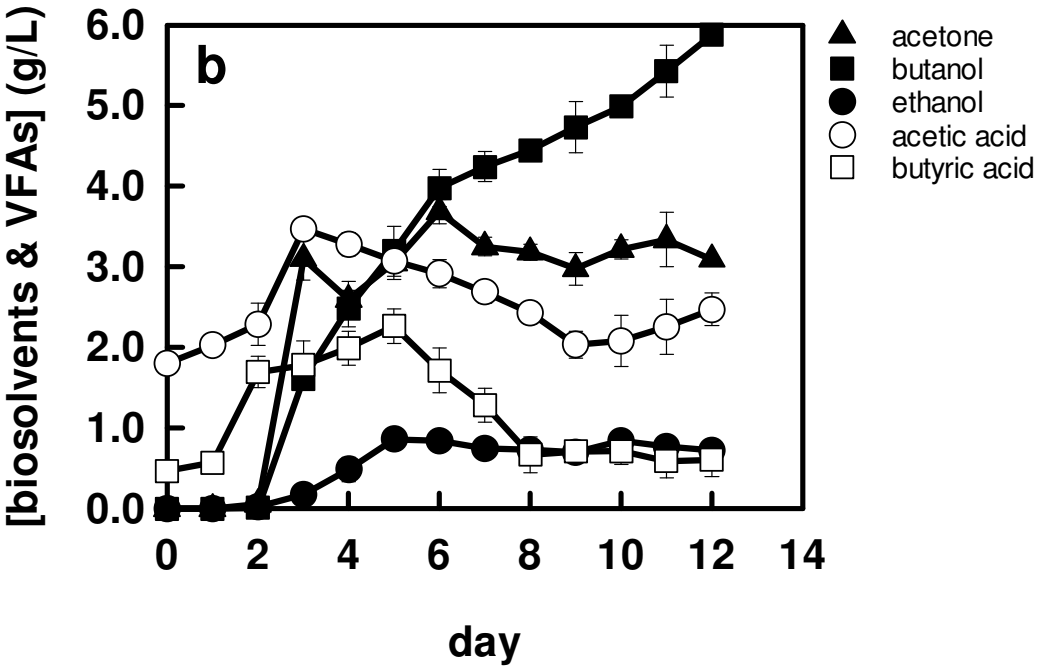


Fig. 3.2. Biosolvents and VFAs production by *Clostridium* species strain BOH3 (10% [v/v] inoculation) with xylose as a substrate. (a) Fed with 30g/L of xylose solely; (b) Fed with 30g/L of xylose and 0.7 g/L of butyrate for butanol enhancement treatment.

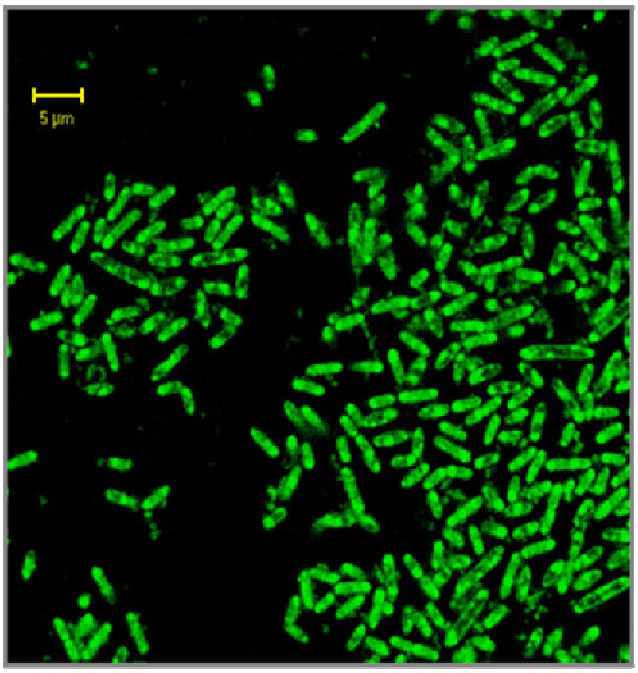


Fig. 3.3. Light microscope examination of culture BOH3 in the early solventogenic phase with glucose as the substrate (day 2)

3.3.2. Enhancement of butanol production

Various treatments were carried out to improve butanol production for culture BOH3. The addition of a precursor compound butyric acid (0.7 g/L) to the glucose-fed culture resulted in an increase of butanol concentration from 4.67 g/L (control) to 5.30 g/L, as shown in Fig. 3.1. However, the heat-shock treated cells did not show improved butanol generation when fed with glucose. As for the xylose-fed cultures, the addition of butyric acid enhanced butanol, acetone, and ethanol production from 4.63 to 5.87 g/L, 2.88 to 3.09 g/L, and 0.61 to 0.72 g/L when compared to the culture without addition of butyric acid (Fig 3.2). The generated hydrogen, however, did not show obvious difference during the above treatments. Noteworthy, keeping an optimal pH range (5.0-5.5) was necessary to ensure constant butanol generation. If the pH value dropped to < 4.5, biosolvents production would be ceased, and butanol generation cannot be recovered even after adjusting the pH back to the optimal range.

By simulating a bioreactor that could retain high amount of biomass, augmented-inoculation treatment (20% inoculation) was performed to improve butanol production in the batch bottle experiments. Results from these bottles shown that butanol reached 7.05 g/L (glucose) and 7.41 g/L (xylose) (Fig.3.4), which was 50.8 and 79.2% higher as compared to the 10% inoculation bottles. Slightly higher hydrogen was also accumulated (6.09 and 4.45 mmol, respectively). Moreover, the augmented inoculation in the glucose-fed BOH3 culture had improved the ratio of acetone and butanol to ethanol (12:19:1), as compared to the commonly observed biosolvent-producing ratio of 3:6:1 by *C. acetobutylicum* or *C. beijerinckii* (Jones and Woods, 1986; Chiao and Sun, 2007). However, the biosolvents ratio did not show distinct difference in the xylose-fed culture BOH3 with or without augmented-inoculation treatment.

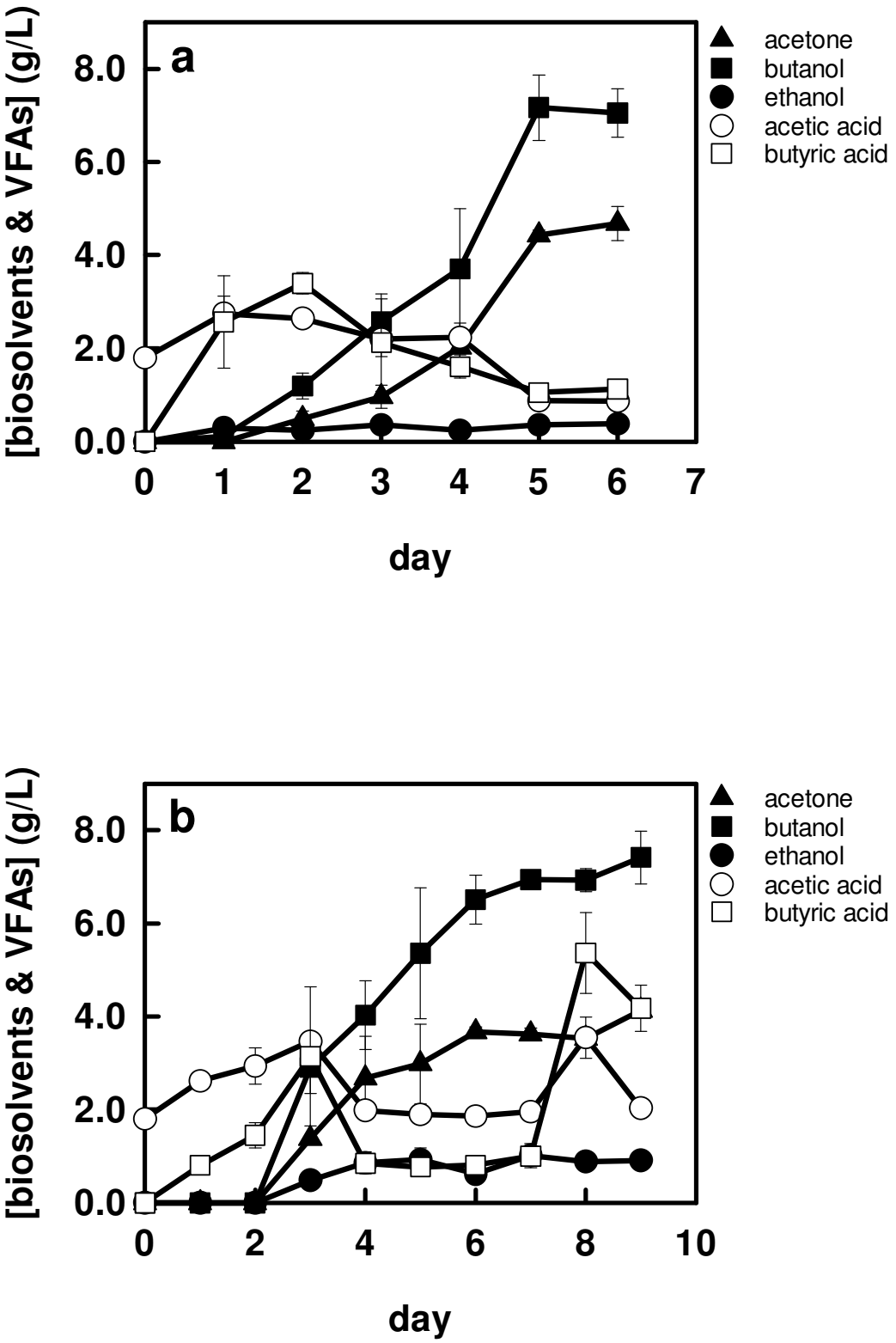


Fig. 3.4. Biosolvents and VFAs production by *Clostridium* species strain BOH3 under augmented-inoculation (20% [v/v]) treatment.
(a) Glucose as a substrate; (b) Xylose as a substrate.

3.3.3. Inhibition study of culture BOH3 on butanol

Previous butanol generation capability was limited by the inhibitory effect of the produced butanol to microorganisms such as *Clostridium* species; therefore, the sustained capability of culture BOH3 on butanol was investigated via spiking with various initial amount of butanol to the experimental bottles. In the presence of 5 or 10 g/L of initially added butanol, culture BOH3 fed on glucose (30 g/L) was still able to produce 6.59 and 6.20 g/L of butanol, though 2 days of lag phase appeared. As compared to the control cultures without the addition of initial butanol, cultures spiked with 5 g/L butanol produced ~6.5% less butanol, while cultures spiked with 10 g/L butanol produced ~12% less. Similarly, in the presence of 5 or 10 g/L of initially-spiked butanol, culture BOH3 fed on xylose shown 5.73 and 5.46 g/L butanol production after 2 days lag, while the control cultures produced 7.41 g/L butanol. For both culture sets fed with glucose or xylose, the butanol production persisted for one week and reached a concentration of 15-16 g/L. The above observations indicated that culture BOH3 could be able to sustain higher amount of butanol (e.g., up to ~16 g/L) than previous wild-type cultures (7.4-11.1 g/L) (Lee et al., 2008).

3.3.4. Yield of butanol

Substrate utilization percentage is an important indicator to determine the efficiency of butanol generation. For culture BOH3, 23.5 and 24.7% of glucose and xylose were converted to butanol in the augmented inoculation, which is comparable to most previous *Clostridium* species (Table 3.1). Culture BOH3 with augmented inoculation shown complete utilization of the supplemented glucose and xylose, indicating that sufficient microbial cells are a prerequisite for efficient carbon conversion to butanol.

Table 3.1. Comparison of biosolvent-yield

Cultures	Amount of utilized substrate	Products concentration			Biosolvent-yield (mass ratio of produced biosolvents and utilized substrate)			References
		Acetone	Butanol	Ethanol	Acetone	Butanol	Ethanol	
	(g/L)	(g/L)			(% g/g)			
C. acetobutylicum	60 g/L glucose	4.35	11.39	1.01	7.25	18.98	1.68	(Bahl et al., 1986)
C. acetobutylicum strain 260	60 g/L glucose	7.38	14.52	0.95	12.30	24.20	1.59	(Ezeji ~~&~~ and Blaschek, 2008)
C. acetobutylicum strain 824	60 g/L glucose	5.24	14.52	0.95	8.73	24.20	1.59	(Ezeji ~~&~~ and Blaschek, 2008)
C. saccharobutylicum strain 262	60 g/L glucose	5.48	11.66	0.48	9.13	19.44	0.79	(Ezeji and Blaschek, 2008)
C. butylicum strain NRLL 592	60 g/L glucose	6.90	14.28	0.38	11.51	23.81	0.63	(Ezeji and Blaschek, 2008)
C. beijerinckii strain BA101	48.9 g/L glucose	6.70	12.70	0.60	13.70	25.97	1.23	(Qureshi and Blaschek, 1999)
C. acetobutylicum strain 260	60 g/L xylose	2.22	9.11	0.67	3.70	15.18	1.11	(Ezeji and Blaschek, 2008)
C. acetobutylicum strain 824	60 g/L xylose	3.33	11.33	0.67	5.56	18.89	1.11	(Ezeji and Blaschek, 2008)
C. saccharobutylicum strain 262	60 g/L xylose	2.00	8.67	0.80	3.33	14.44	1.33	(Ezeji and Blaschek, 2008)
C. butylicum strain NRLL 592	60 g/L xylose	0.67	5.11	0.22	1.11	8.52	0.37	(Ezeji and Blaschek, 2008)
***C. butyricum* strain BOH3**	**30 g/L glucose**	**4.68**	**7.05**	**0.38**	**15.61**	**23.51**	**1.27**	**this study**
***C. butyricum* strain BOH3**	**30 g/L xylose**	**4.12**	**7.41**	**0.91**	**13.75**	**24.71**	**3.03**	**this study**

In the augmented-inoculation treatments on glucose-fed cultures, the cells increased from 0.21 to 0.93 g cellular protein/L, which appeared to be similar to the culture spiked with initial high amount of butanol (~10 g/L). On the other hand, the BOH3 cells increased from 0.21 to 0.71 g cellular protein/L when fed with xylose. The butanol yield for culture BOH3 was calculated to be 8.10 and 8.51 g/g cellular protein after consuming 30 g/L glucose and xylose.

3.3.5. Utilization of cellulose and xylan

Bioconversion of cellulose and xylan into bioenergy could make the use of renewable feedstock feasible. In the presence of xylan and cellulose, culture BOH3 was capable of producing fuels such as hydrogen and VFAs (predominantly acetic and butyric acids) under mesophilic conditions (Fig. 3.5). The quantities of acetic acid and butyric acid generated from cellulose reached as high as 1.40 and 0.71 g/L.

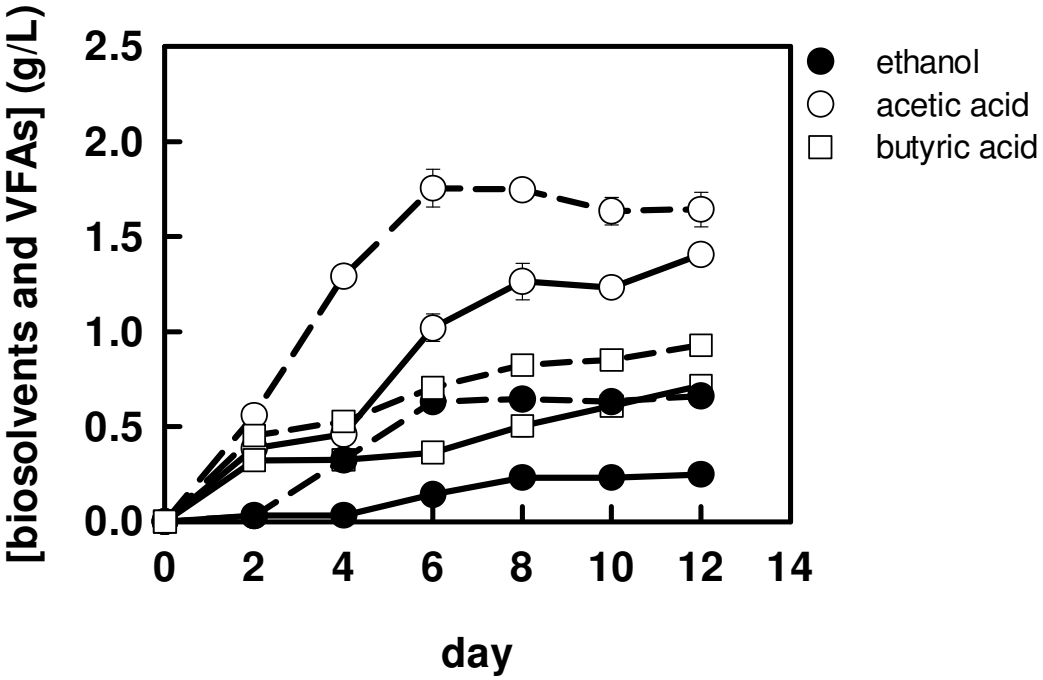


Fig. 3.5. Ethanol and VFAs production from 10 g/L of substrate by *Clostridium* species strain BOH3. (a) Cellulose as substrate (solid lines); (b) Xylan as substrate (dotted lines).

Under similar conditions, the amounts of acetic acid and butyric acid produced from xylan reached 1.64 and 0.92 g/L, respectively. Solventogenic activity was also observed, and butanol was produced in a concentration of approximately 0.02 g/L for both substrates while ethanol was produced at 0.65 and 0.24 g/L in the presence of xylan or cellulose.

Additionally, hydrogen was also generated to an amount of 2.47 and 1.93 mmol from cellulose and xylan (Fig. 3.6), and the rest of the gas phase was accounted by CO_2. Culture BOH3 shown a yield of 0.97 mmol hydrogen/g cellulose and 0.85 mmol hydrogen/g xylan, which was calculated by using the total cumulative hydrogen per gram of initial cellulose or xylan over 12 days. The initial cellular protein concentrations for cellulose- and xylan- fed cultures were 0.07 g/L, which increased to 0.25 and 0.26 g/L after 12 days of incubation.

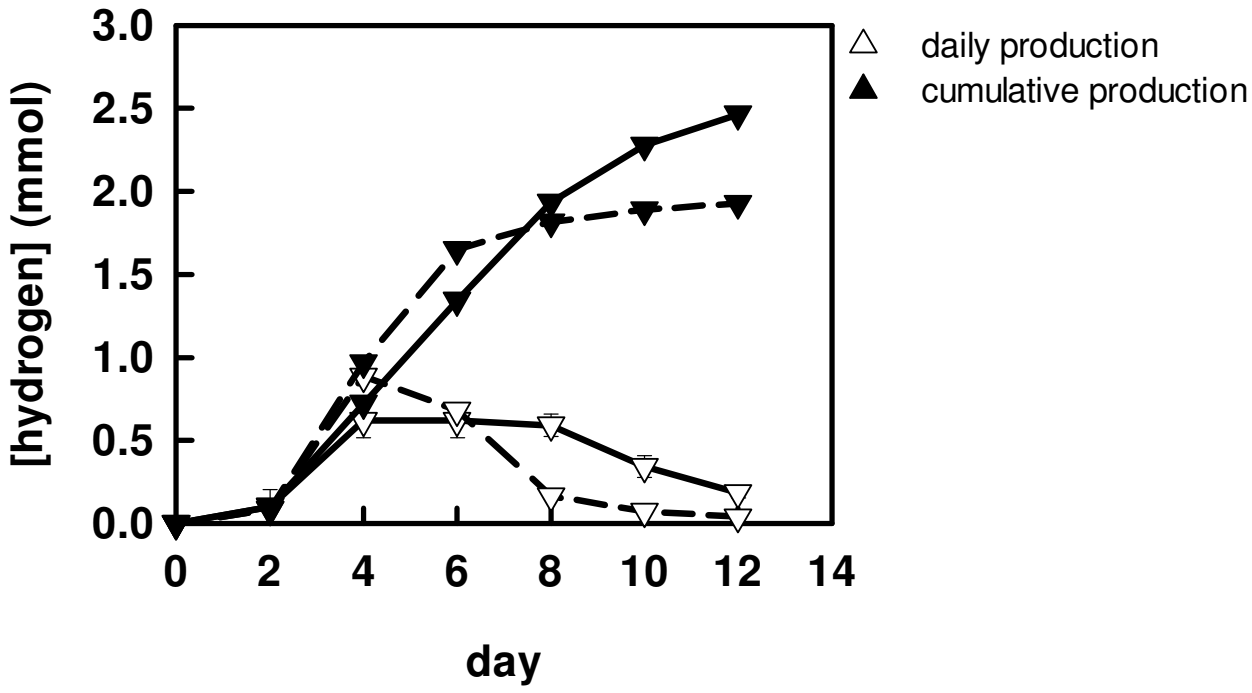


Fig. 3.6. Hydrogen production from 10 g/L of substrate by *Clostridium* species strain BOH3. (a) Cellulose as substrate (solid lines); (b) Xylan as substrate (dotted lines).

3.4. Discussion

In this study, a new *Clostridium* species strain BOH3 is obtained and discovered to be capable of fermenting both glucose and xylose to produce butanol at concentrations comparable to other widely studied biosolvents-producing strains such as *C. acetobutylicum* and *C. beijerinckii* (Jones and Keis, 1995). Additionally, culture BOH3 shows advantage of high resistance against butanol inhibition (up to ~17 g/L). Considering that 7.4-11.1 g/L of butanol was inhibitory to the fermentative processes of other solventogenic strains among the *Clostridia* (Lee et al., 2008), strain BOH3 demonstrated a marked tolerance against butanol by producing additional 5.5-6.6 g/L of butanol despite an initial butanol stress of 10 g/L. The resistance against butanol suggests that strain BOH3 may have the potential to be operated at higher butanol concentrations in an industrial chemostat, thus bringing economic benefits. During the enhancement of culture BOH3's butanol yield, high initial cells density seems to be more efficient than the addition of butyric acid. This can be attributed to the promoted higher consumption of the substrates (e.g., full utilization of glucose/xylose in the augmented inoculation) by the large number of cells. Notably, butanol can only be generated when the cells enter sporulation process but it discourages cells growth. Therefore, balancing cells growth and sporulation timing is a key factor to influence the flow of substrate carbon to cells growth or butanol generation. Most importantly, keeping the cells in pre-sporulation state (optimum pH at 5.5) is necessary in maximizing the carbon conversion to final products, since cells cannot reverse from spores to pre-sporulation as observed in this study and previous studies (Lee et al., 2008).

Moreover, culture BOH3 also distinguishes itself by directly utilizing crystalline cellulose and xylan to generate significant amounts of hydrogen, VFAs, and trace amount of biosolvents (e.g., ethanol and butanol) under mesophilic conditions without enzymatic hydrolysis of the polymerized substrates. Thus, culture BOH3 possesses cellulolytic,

xylanolytic, and solventogenic properties, which have yet to be shown in previous reports. Besides cellulose, culture BOH3 can convert xylan to hydrogen and VFAs effectively, which is an important feature due to the considerable proportion of xylan (~8-23% by dry weight) in lignocellulosic biomass, particularly in old woody biomass (Sun and Cheng, 2002; Mosier et al., 2005). Since there are limited studies on the utilization of xylan, the results in this study differentiate *Clostridium* species strain BOH3 from other butanol- and hydrogen- producing species by possessing xylanase-producing and xylose-utilizing capabilities. Furthermore, when utilizing both cellulose and xylan, the hydrogen yields of culture BOH3 are comparable to or higher than other studies employing various types of bacterial strains (e.g., *C. pasteurianum* and *Enterococcus gallinarum* G1) at 35-37°C (Lo et al., 2008; Wang et al., 2009) and the thermophilic *C. thermocellum* strain JN4 (Liu et al., 2008). Comparing with another thermophilic bacteria – *Anaerocellum thermophilum strain* DSM 6725 (Yang et al., 2009), culture BOH3 produced less hydrogen (2.46 and 1.92 mmol hydrogen from 10 g/L of cellulose and xylan versus 5.50 mmol hydrogen from 5 g/L of cellulose and xylan). However, the acetic acid generated (23.4 and 27.3 mM) by culture BOH3 was almost eight- to nine-fold of that in strain DSM 6725 (~3 mM), indicating the former's unique pathways. Noteworthy, during the hydrolysis and hydrogenesis of cellulose- and xylan- fed cultures, keeping an optimum pH (7.0-7.2) is necessary to maintain reasonable enzymatic activity (e.g., hydrogenase), since the activity of the enzyme is inhibited at low pH (Khanal et al., 2004). The higher amount of VFAs produced from polysaccharides by culture BOH3 paves the way for future direct conversion of sustainable polymeric cellulose and xylan into biofuels.

3.5. Conclusion

In this study, a new *Clostridium* species strain BOH3 is discovered to ferment both glucose and xylose to produce butanol at concentrations comparable to other widely studied biosolvents-producing strains. Culture BOH3 also distinguishes itself by directly utilizing cellulose and xylan to generate significant amounts of hydrogen, VFAs, and trace amounts of ethanol under mesophilic conditions without exogenous enzymes. The VFAs produced from polysaccharides by culture BOH3 paves the way for future direct conversion of sustainable biomass into biofuels. In summary, culture BOH3 is promising due to its: (i) capability to survive under high concentrations of butanol; (ii) direct fermentation of (hemi)cellulosic materials to hydrogen and butanol precursors; and (iii) potential to be optimized in continuous systems for better bioenergy yields.

CHAPTER 4

Optimization of butanol generation by *Clostridium* species strain BOH3

Abstract

The high cost of yeast extract-peptone (YEP), which are required for butanol production, has greatly increased the production cost of fermentative butanol. Here we report that the omission of YEP was amenable through the modification of substrate dosing method, incorporated with the high cell density inoculation, to improve butanol production by a wild-type solventogenic *Clostridium* strain BOH3. Culture BOH3 did not show solventogenic performance when it was bulk dosed with 90 g/L of glucose or xylose, while it produced 14.17 g/L and 11.69 g/L of butanol, respectively in 12 days, when it was stepwise dosed with 30 g/L of glucose or xylose each. A further enhancement by coupling the stepwise dosing of substrate and inoculation of higher initial cell density as augmented-inoculation setup resulted in a reduction in fermentation time to 10 days for glucose and xylose setups. Addition of YEP improved the hydrogen production for glucose and xylose setups by 52.32% and 79.67%, respectively. Nonetheless, the abovementioned enhancements did not alter the maximum production of butanol due to the butanol toxicity limit. Therefore, dosing the substrate in a stepwise mode and implementation of high cell inoculation improved the butanol fermentation rate and hydrogen production yield in the absence of YEP.

Keywords: Augmented-inoculation; Butanol; *Clostridium*; Stepwise dosing; Yeast extract-peptone

4.1. Introduction

Biological production of butanol has recently received scientific attention, since it is an emerging form of fuel. Butanol offers advantages over ethanol due to its properties, such as immiscibility with water, less hydroscopic, non corrosive, lower vapor pressure, lower heat of vaporation, ability to be blended in higher proportions with gasoline, higher energy density, and ability to be directly used in gasoline engines without modification (Ni and Sun, 2009). From a bioprocess standpoint, employing genetically modified microorganisms is less preferable than the wild-type ones, as their unstable performance due to plasmid excision may lead to the deterioration of butanol-producing capability within fermentation cycles (Heap et al., 2007). Furthermore, there is potential for genetic information to be transferred between individuals, both vertically (parent-offspring) and horizontally (individual-individual) that might generate unknown impacts to the ecological balance. For instance, plasmids, the extrachromosomal pieces of circular DNA that carry genes conferring desirable and undesirable traits may be freely exchanged between individuals, including between different species (Levin and Stewart, 1977; Lewin, 1982; Myhr and Traavik, 1999). Hence, the discovery of a novel and enhanced wild-type microorganism capable of withstanding the substrate inhibition and product inhibition of butanol while maintaining the high cell density during the solventogenic stage would be an important milestone in achieving high performance of butanol production. Furthermore, modification of the reactor operation mode to manipulate the process can further complement the enhanced performance of the microrganisms.

Solventogenic fermentation has a distinct characteristic, in term of the depletion of cell density during the butanol production stage. A rapid increase of cell density occurs during the acid production or acidogenic stage, which is coupled with ATP formation, followed by the high amount of hydrogen and carbon dioxide generation. The onset of

butanol production in the solventogenic stage is indicated by the re-assimilation of acids to butanol, with other solvents in lesser amounts (Dürre, 2008). The decrease of cell density in the fermentation reactor occurs due to the presence of produced butanol, in the range of 7.4–11.1 g/L. This leads to the deactivation of ATPase, sugar uptake hindrance, membrane destabilization, transport process disruptions, and finally to cell growth inhibition (Lee et al., 2008). The decrease of cell density in the solventogenic stage can be addressed by the provision of higher ratio between initial cell density and substrate. This process manipulation has been performed/conducted in previous studies that employed the immobilized cell or recycled cell system, in order to maintain the high density of cells (Lee et al., 2008). Notably, the entire cells in the immobilized cell system are not in the solventogenic stage, as the probability of some cells are in the inactive spore condition (Asif and Muneer, 2007). This resulted in the improvement of fermentation yields and rates, but the specific productivity often declines with the increase of cell density.

Besides employing higher cell density, the method of substrate dosing also plays an important role. Bulk dosing of substrate might generate inhibition to the microbes as excessive amount of substrate may lead to the rapid production of acids due to the low ratio between the initial cell density and substrate (Fond et al., 1985; Ezeji et al., 2004). In the absence of a proper pH control, cell enters plateau growth phase followed by death phase, due to the rapid decrease of pH. Gradual feeding of the substrate, i.e., stepwise dosing method, is expected to reduce the acids production rate, thus allowing the microbes to re-assimilate the acids to butanol. This method avoids any lag phase due to the substrate inhibition and provides a stable condition for the cells to adapt to the incoming substrate and produce acids simultaneously.

Reactor operation is mostly adjusted to perform butanol production at the controlled pH range of ~5.0–6.0 (Fond et al., 1985; Johansson and Azar, 2007). Notably, solventogenic

Clostridia also has the distinct ability to regulate the pH, by maintaining the pH difference between internal cells pH (pHi) and external cells pH (pHe) (Rathmann et al., 2010). pH control helps to maintain this pH difference, because of some cultures' inability to self-regulate the pH. Nevertheless, the absence of pH control can reduce the investment and operation costs for the butanol production at the industrial-scale.

In this study, solventogenic fermentation of a mesophilic wild-type microbe – designated *Clostridium* species strain BOH3, was investigated. Combination of stepwise dosing of substrate and high cell density inoculation were performed, in accordance with the absence of external pH control. In addition, influence of YEP addition on culture BOH3 was also investigated, in an effort to optimize the reactor conditioning, specifically for the wild-type cultures.

4.2. Materials and Methods

4.2.1. Microorganism, culture activation, and inoculation

Laboratory stock of *Clostridium butyricum* strain BOH3 was activated at 35 ^{o}C in a 125-rpm shaking incubator for 48 hours, prior to the fermentation experiment. The activation medium was 36-mL of mineral salt medium in 60-mL bottles with 10% culture inoculation (v/v) and 10 g/L of glucose as the substrate. After 48 hours of activation, 4-mL of active culture BOH3 (10% [v/v]) was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was decanted carefully in an anaerobic chamber, and the centrifuged cell pellet was re-dissolved by 4-mL of mineral salt medium, prior to the inoculation for the fermentation study. Experiment was conducted in triplicate bottles that contained 36-mL reduced mineral salts medium in a 60-mL serum bottle, supplemented with Wolin solution

(Wolin et al., 1963), and if necessary, with 1 g/L of YEP addition. Fermentation course was conducted in shaker incubator at 35°C controlled-temperature setup with a 125-rpm shaking speed. The experiments with this inoculation method are termed as control.

A modified inoculation setup, namely augmented-inoculation, was aimed to increase the amount of initial cell inoculum without excessively diluting the fermentation broth. 8-mL of active culture was aliquoted and centrifuged to separate the cell, as abovementioned method. This doubled amount of cell was dissolved in 4-mL of mineral salt medium for the inoculation, thus kept the constant inoculation size, 10% [v/v].

4.2.2. Substrate dosing

Substrate dosing was performed by gradual addition of 30 g/L of glucose or 30 g/L of xylose to achieve the final concentration of 90 g/L. Subsequent addition of 30 g/L of glucose or xylose was performed when the produced gas volume along the fermentation course has reached ~60-70% of the maximum achievable gas volume production from the previous 30 g/L of substrate addition. A preliminary experiment was conducted to measure the maximum achievable gas volume production from 30 g/L of glucose or xylose.

4.2.3. Chemical analysis

Refer to subchapter 3.2.3.

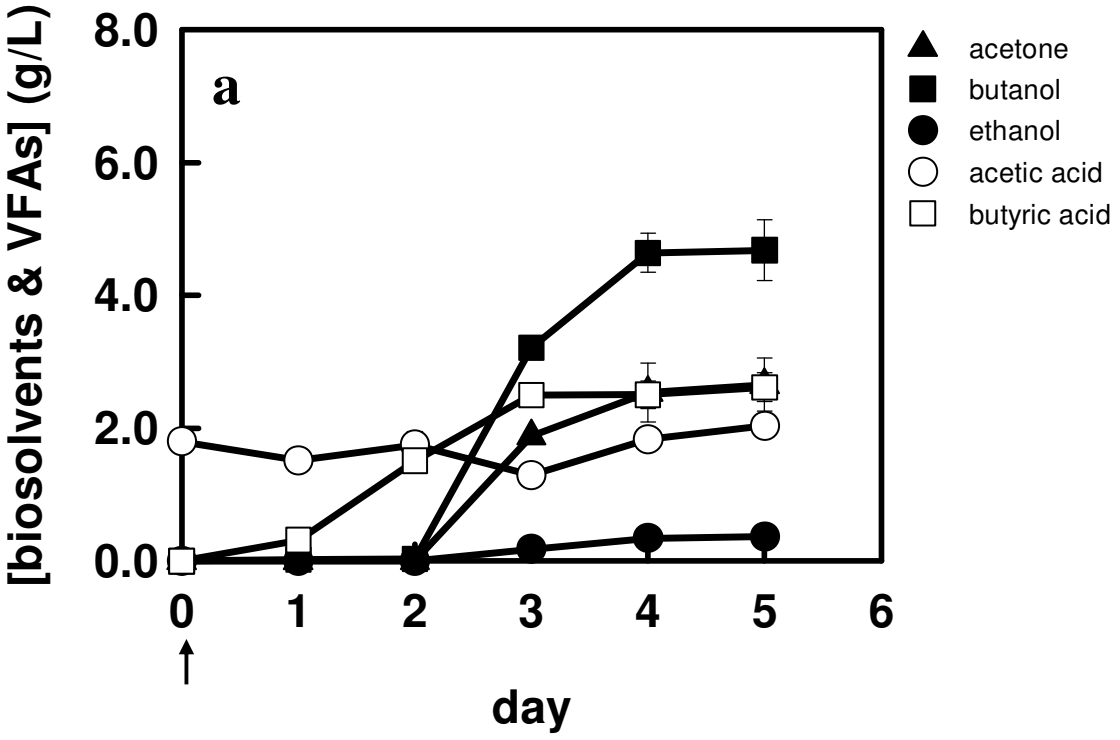
4.3. Results

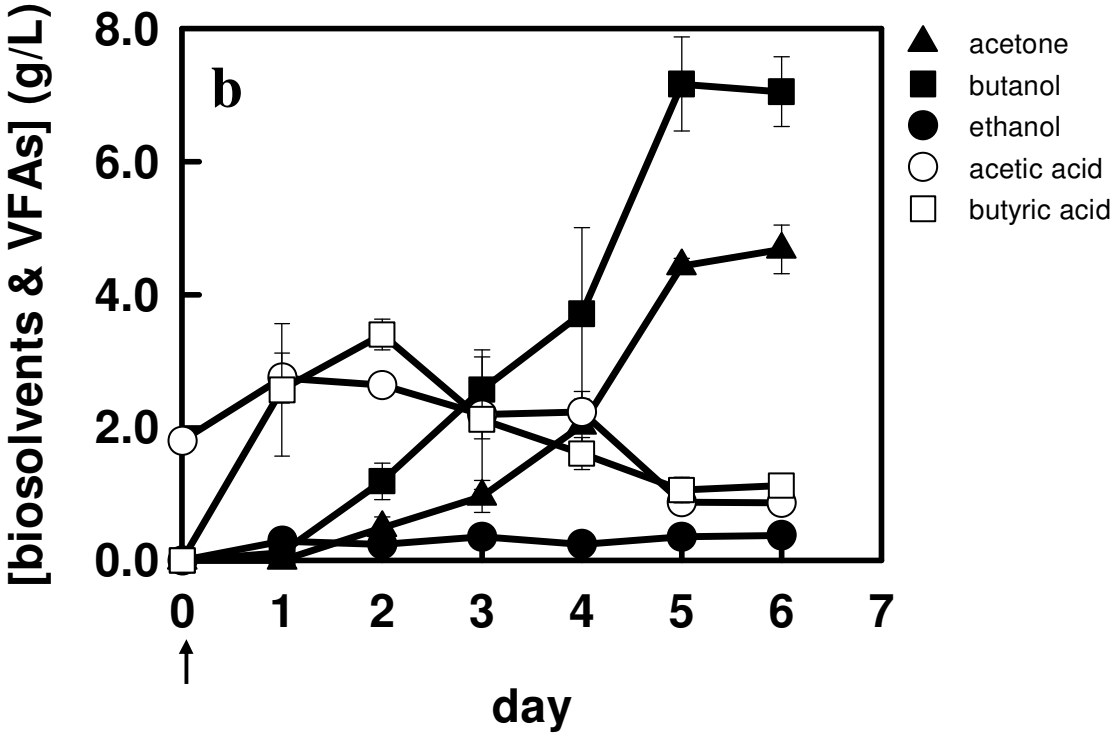
4.3.1. Investigation on augmented-inoculation setup

The investigation was initiated by assessing the performance of culture BOH3 to convert low amount of substrate, 30 g/L of glucose or xylose, to butanol. The ability of this culture to produce 4.67 g/L of butanol within 5 days from glucose and 4.63 g/L of butanol within 12 days from xylose confirmed its solventogenic capabilities (Fig. 3.1.). Hydrogen as

another energy-rich product was also produced from medium containing glucose and xylose, with the cumulative amounts of 5.40 mmol and 4.22 mmol, respectively. This initial notable solventogenic performance of culture BOH3, serves as a control setup.

The latter condition, such as augmented-inoculation, employed 0.14 g cellular protein/L as the initial cell inoculum density, in comparison to 0.07 g cellular protein/L for the control setup. Superiority of augmented-inoculation setup was showed by a faster fermentation rate and higher final concentration of produced butanol. Fermentation ceased within 6 and 9 days for glucose and xylose as the substrates, with the final concentrations of butanol of 7.05 g/L and 7.41 g/L (Fig 4.1). However, lower amount of cumulative hydrogen was observed, 5.14 mmol and 4.03 mmol, respectively.




b
[biosolvents & VFAs] (g/L)
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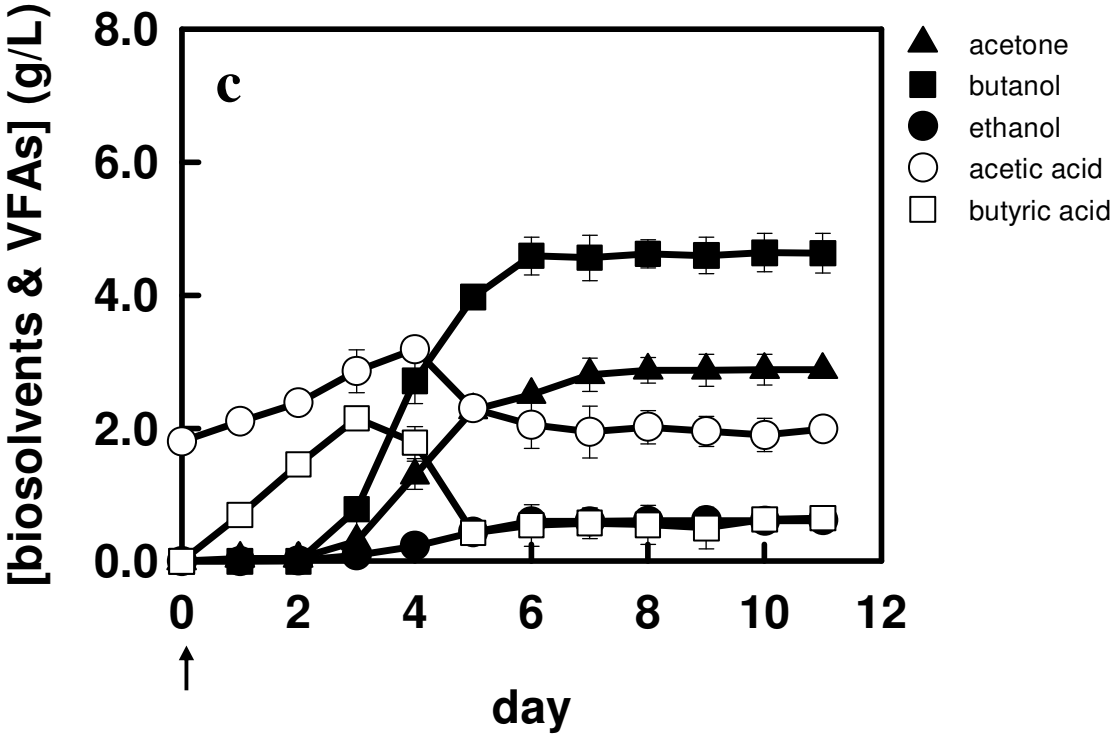
6.0
4.0
2.0
0.0
0
1
2
3
4
5
6
7
day
acetone
butanol
ethanol
acetic acid
butyric acid


c
[biosolvents & VFAs] (g/L)
8.0
6.0
4.0
2.0
0.0
0
2
4
6
8
10
12
day
acetone
butanol
ethanol
acetic acid
butyric acid

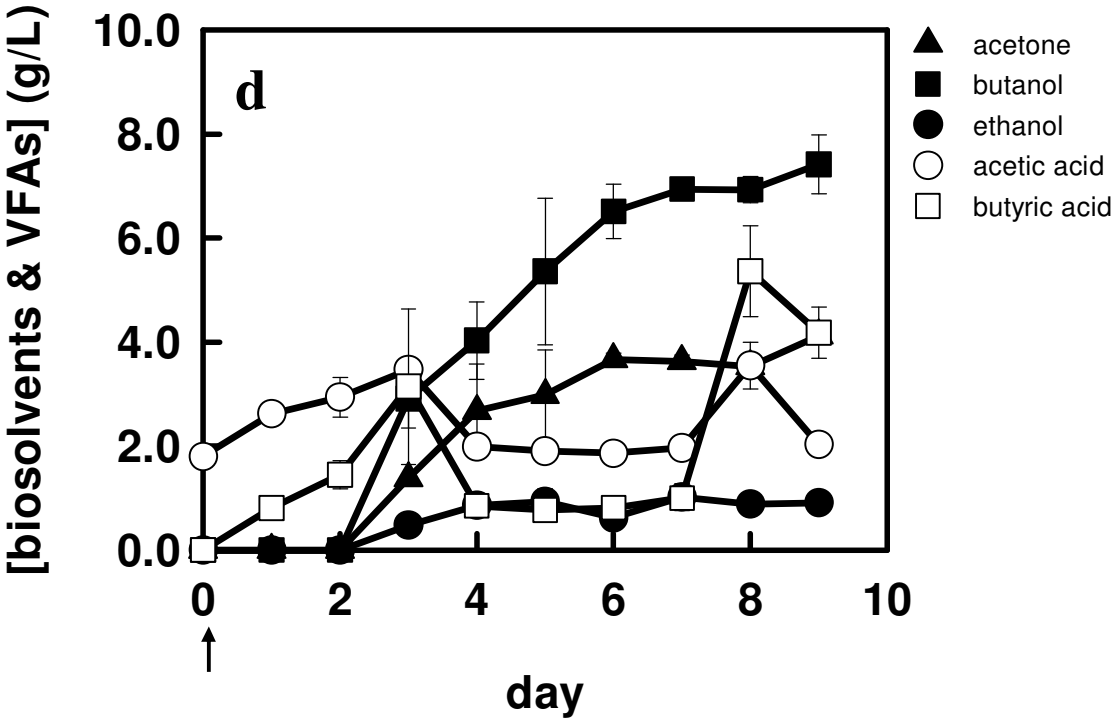


Fig. 4.1. Production of biosolvents and VFAs by *Clostridium* species strain BOH3 from 30 g/L of substrate. (a) Glucose-fed, control setup; (b) Glucose-fed, augmented-inoculation setup;(c) Xylose-fed, control setup; (d) Xylose-fed, augmented-inoculation setup
Note : ↑ denotes the substrate addition point

Due to the higher amount of fermentation products observed, it was deduced that higher substrates utilization took place. In comparison, 2.18 g/L of glucose and 1.45 g/L of xylose were still detected for the control setup, while undetected amounts of the remaining glucose and xylose substrates for augmented-inoculation setup. Therefore, subsequent experiments employed higher initial cell density for the inoculums size.

4.3.2. Combination of augmented-inoculation setup and stepwise dosing of substrate

The presence of 30 g/L of glucose or xylose in bulk dose did not show substrate inhibition. This indicates that higher amount of bulk-dosed substrates can be investigated to produce higher butanol concentrations. Previous studies showed the bulk dose of 60 g/L of glucose as the baseline to achieve maximum production of butanol by *Clostridium* (Assobhei

et al., 1998; Ezeji et al., 2007b; Ezeji and Blaschek, 2008). However, culture BOH3 showed a lag phase of 4 days when the culture was bulk-dosed with 60 g/L of glucose or xylose, followed by the insignificant production of acetic acid and butyric acid at day 5. Culture BOH3 also did not re-assimilate the produced minute quantities of acids to butanol after day 5, which indicated the absence of the glucose or xylose utilization and cell growth in the following days. This result showed that the bulk dose (60 g/L) of glucose or xylose was the intolerable amount of substrate concentration for culture BOH3. Therefore, subsequent studies were conducted by spiking the substrate at the concentration lower than 60 g/L. For example, 30 g/L each was performed in a stepwise mode until the maximum production of butanol concentration was achieved. A preliminary investigation on culture BOH3 showed ~500–600-mL of gas can be produced from the fermentation of 30 g/L of glucose or xylose (data not presented). This cumulative amount of produced gas was usually observed when substrate depletion has been achieved. Another dose of 30 g/L of glucose or xylose in a stepwise method was spiked to the culture, and the gas production from the fermentation process has achieved ~350–420-mL, or 60–70% from the total maximum producible gas.

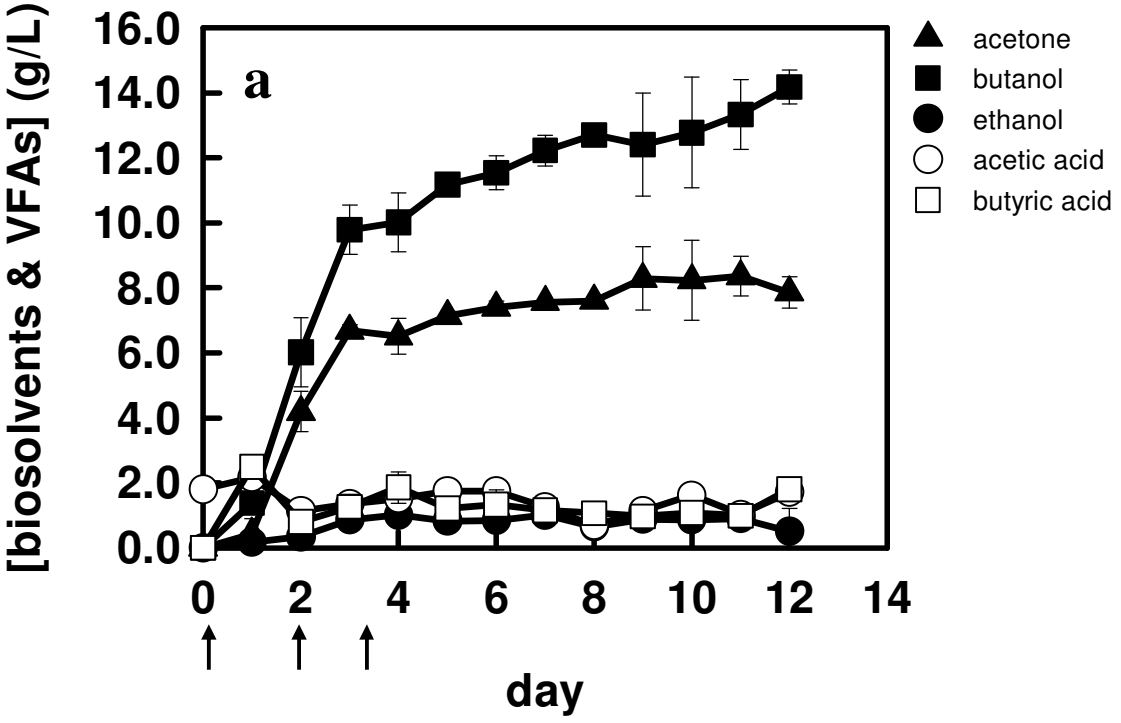
Control in the medium containing glucose setup showed that 14.17 g/L of butanol can be produced within 12 days from 90 g/L of glucose. The 90 g/L of glucose was dosed in a stepwise mode of 30 g/L each, at day 0, day 2, and day 3 showed slightly lower produced butanol, 13.70 g/L, within shorter time of 10 days, dosed at day 0, day 1, and day 7. A higher cumulative hydrogen production was observed compared with control, 13.68 mmol and 12.10 mmol.

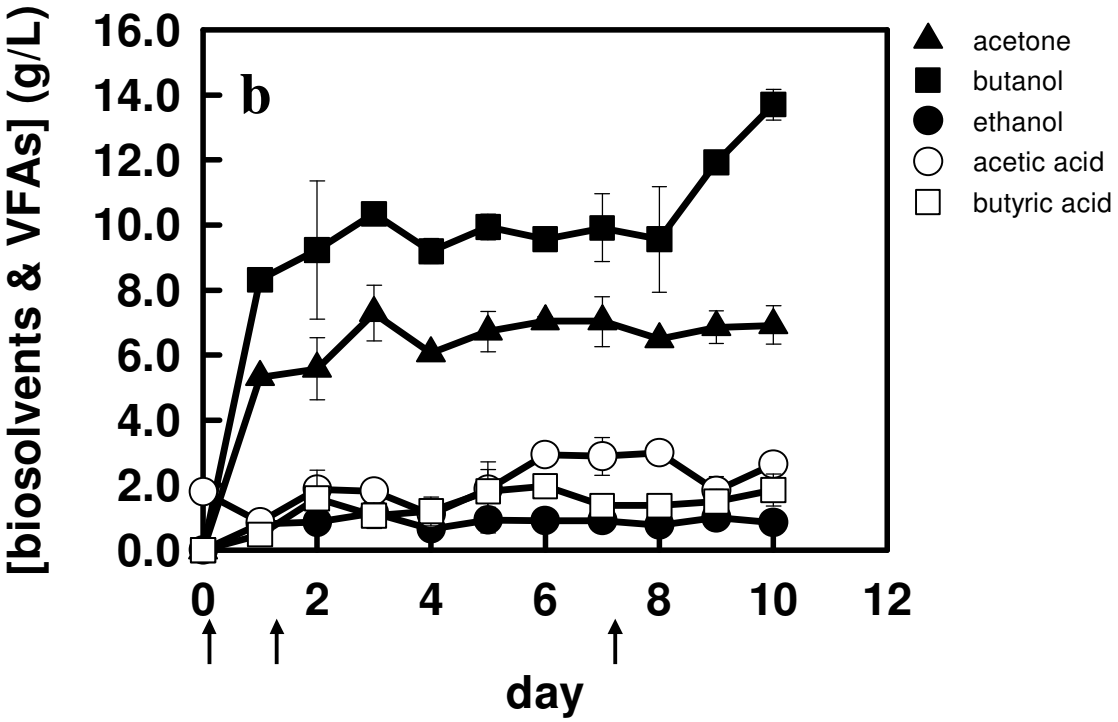
In comparison to medium containing xylose, the control setup produced 11.69 g/L of butanol within 12 days, in which 30 g/L of xylose each was dosed in stepwise method at day 0, day 3, and day 7. Augmented-inoculation setup was able to complete the fermentation course within 10 days, with a slightly lower butanol production (10.02 g/L) when xylose was

dosed at day 0, day 3, day 6 (Fig 4.2). Higher amount of cumulative hydrogen was obtained for augmented-inoculation setup compared with control, 9.74 mmol and 8.99 mmol. Neither glucose nor xylose was detected at the end of fermentation for augmented-inoculation setup, while control setup still showed 1.88 g/L of glucose and 2.53 g/L of xylose as the unutilized amount of substrates.

4.3.3. Combination of augmented-inoculation setup and stepwise dosing methods, enhanced with yeast extract-peptone addition

Addition of YEP was performed in most of butanol production studies, as complex nitrogen source for the culture (Fond et al., 1985; Assobhei et al., 1998; Qureshi and Blaschek, 1999; Johansson and Azar, 2007; Ezeji and Blaschek, 2008). However, the spiking of costly YEP also increases the butanol production cost (Parekh et al., 1999), thus its avoidance would be beneficial to achieve an economically affordable butanol production. In this part of the study, the effect of YEP addition was investigated, in comparison with combination of augmented-inoculation setup and stepwise dosing of substrate mode.




b
[biosolvents & VFAs] (g/L)
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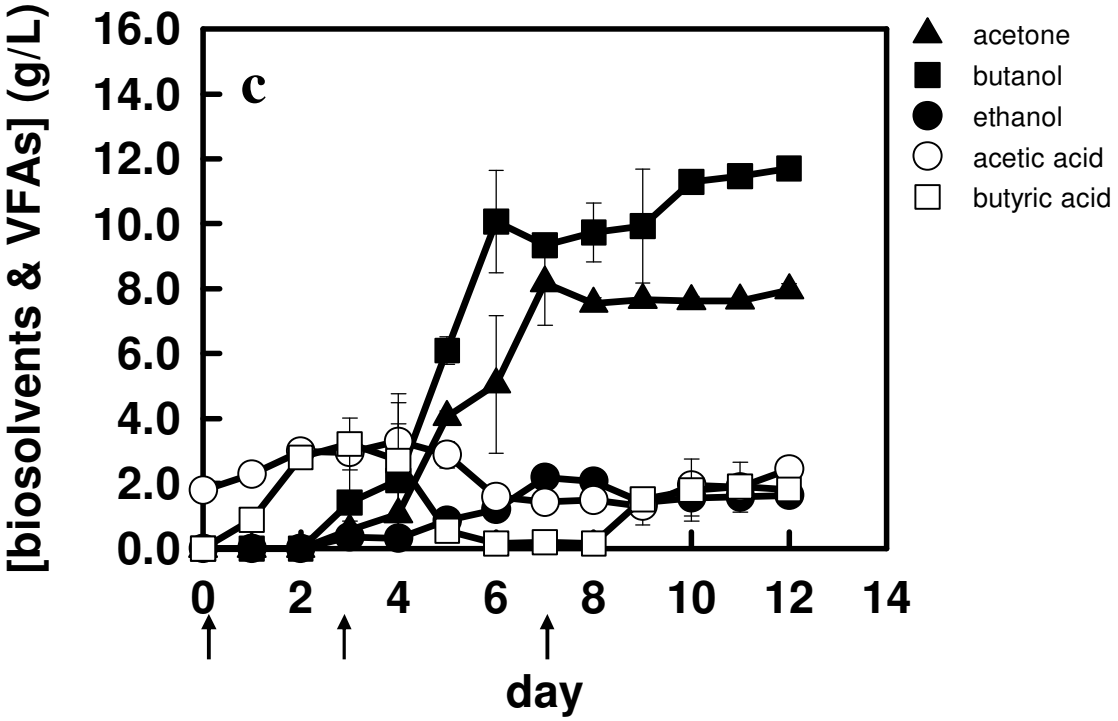
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
2
4
6
8
10
12
day
acetone
butanol
ethanol
acetic acid
butyric acid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[biosolvents & VFAs] (g/L)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
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4
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8
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day
acetone
butanol
ethanol
acetic acid
butyric acid

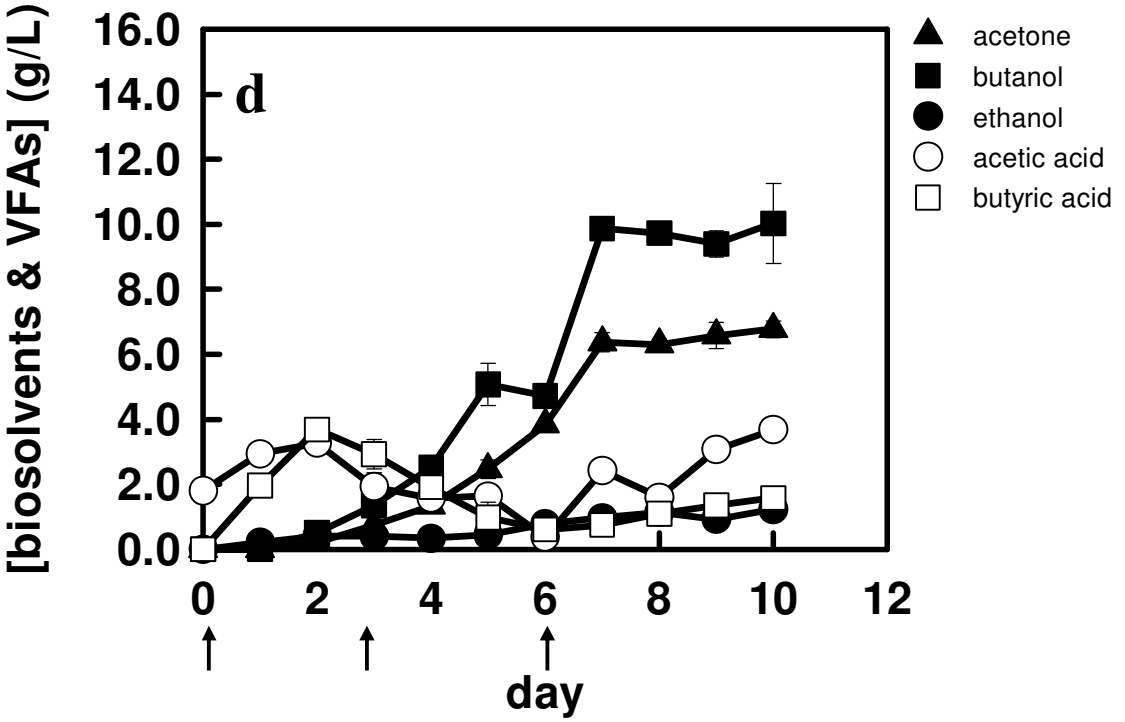
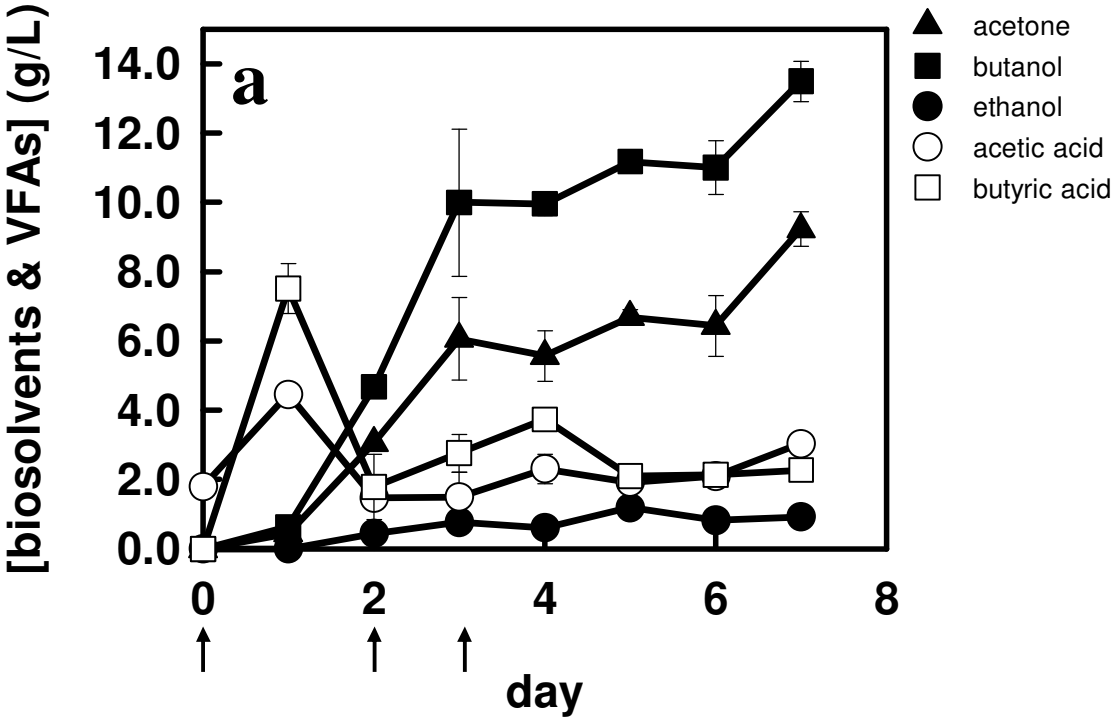


Fig. 4.2. Enhanced production of biosolvents and VFAs by *Clostridium* species strain BOH3 from 90 g/L of substrate by augmented-inoculation and stepwise dosing of substrate. (a) Glucose-fed, control setup, stepwise dosing; (b) Glucose-fed, augmented-inoculation setup, stepwise dosing; (c) Xylose-fed, control setup, stepwise dosing; (d) Xylose-fed, augmented-inoculation setup, stepwise dosing.
Note : ↑ denotes the substrate addition point

Augmented-inoculation setup reduced the fermentation time, thus increased the fermentation rate compared with the control. Further investigation was performed by combining augmented-inoculation setup and stepwise dosing of substrate, together with YEP addition. Notably, results showed YEP addition insignificantly improved the final concentration of the produced butanol, however, a shorter fermentation time and higher production of hydrogen were observed. A total of 90 g/L of substrate was stepwisely dosed (individually of 30 g/L) at day 0, day 2, and day 3 for glucose-fed medium that produced 13.49 g/L of butanol within 7 days. 30 g/L of xylose each was individually dosed at day 0, day 3, and day 6 produced 11.56 g/L of butanol within 11 days (Fig 4.3). Hydrogen production improved significantly through YEP addition, which reached 18.44 mmol and

16.16 mmol for medium containing glucose and xylose, respectively, and both substrates were fully consumed at the end of fermentation. Cellular protein concentrations increased to 0.17 g/L and 0.16 g /L for glucose and xylose-feds at the end of fermentation, in comparison with 0.15 g/L for both substrates in the starting of fermentation.



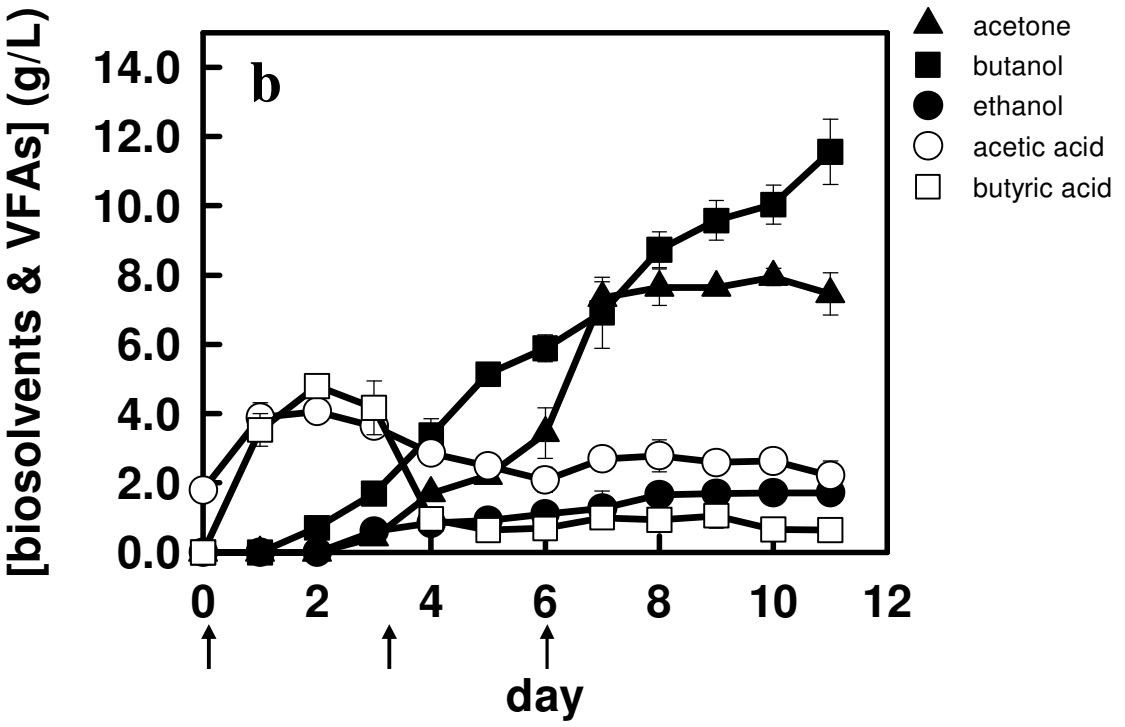
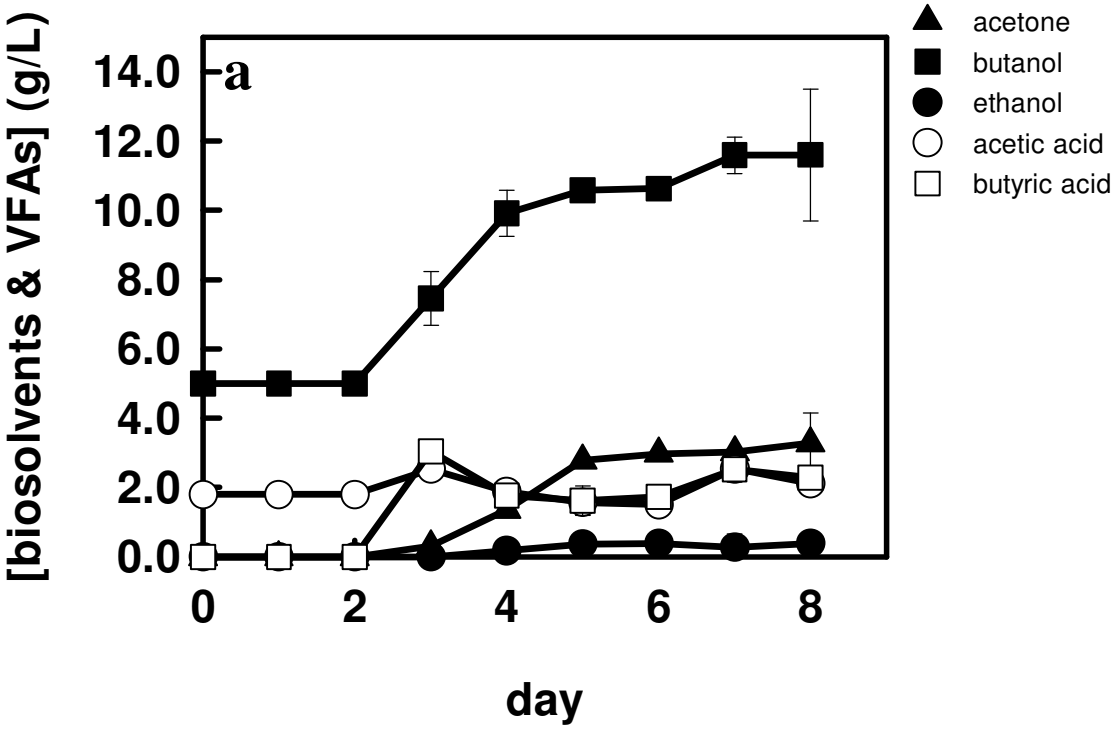
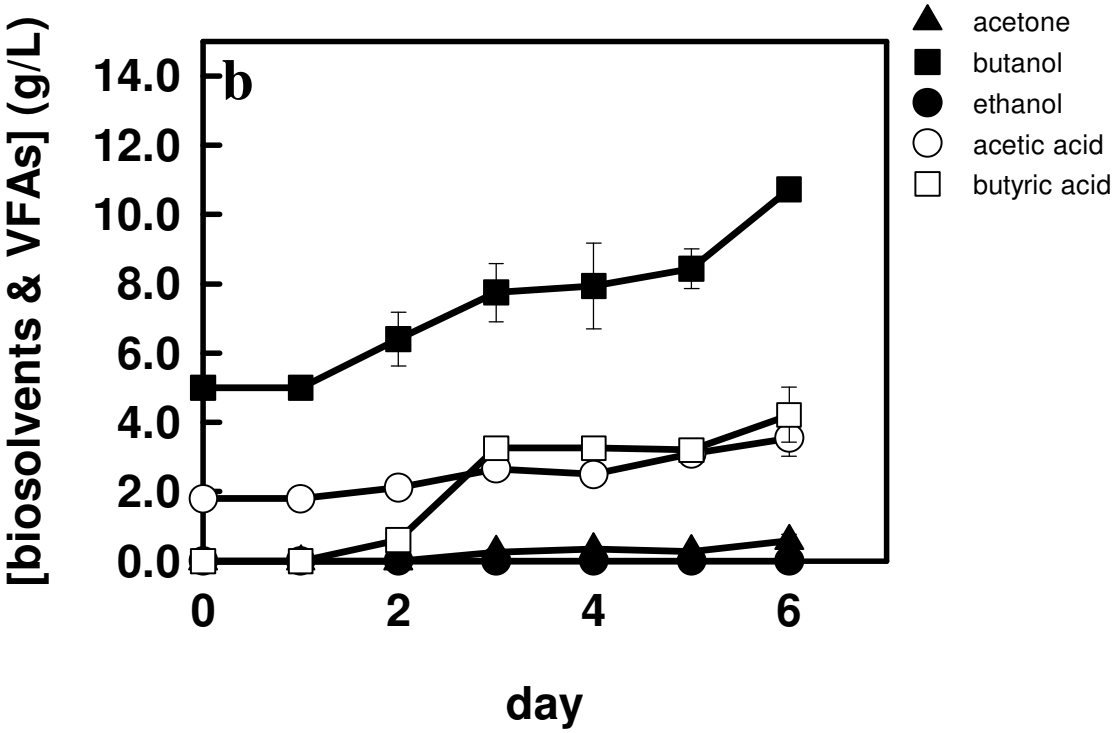


Figure 4.3. Enhanced production of biosolvents and VFAs by *Clostridium* species strain BOH3 from 90 g/L of substrate, with the presence of yeast extract-peptone (YEP). (a) Glucose-fed, augmented-inoculation setup, stepwise dosing, YEP addition; (b) Xylose-fed, augmented-inoculation setup, stepwise dosing, YEP addition.
Note : ↑ denotes the substrate addition point

The final concentration of the produced butanol has never exceeded 15 g/L. This was due to the butanol toxicity effect as product inhibition to the culture, thus diminishing the culture growth. The growth of solventogenic *Clostridia* was severely affected due to the presence of 7.4–11.1 g/L of butanol (Lee et al., 2008), even for the control or augmented-inoculation setup. In order to confirm this hypothesis, a butanol toxicity study was conducted to investigate the cause for the stagnating production of butanol when a certain butanol concentration was reached. With augmented-inoculation setup, culture BOH3 was able to produce ~5.5–6.5 g/L of butanol, in spite of the presence 5-10 g/L of exogenous butanol (Fig 4.4.). High cell density as performed in augmented-inoculation setup was expected to

minimize the effect of toxic butanol, thus could minimize the shear effect of butanol to the cell (Chang et al., 1994; Dürre et al., 2008; Lee et al., 2008) . A higher concentration of spiked-butanol of 15 g/L showed a completely inhibition to the culture growth.





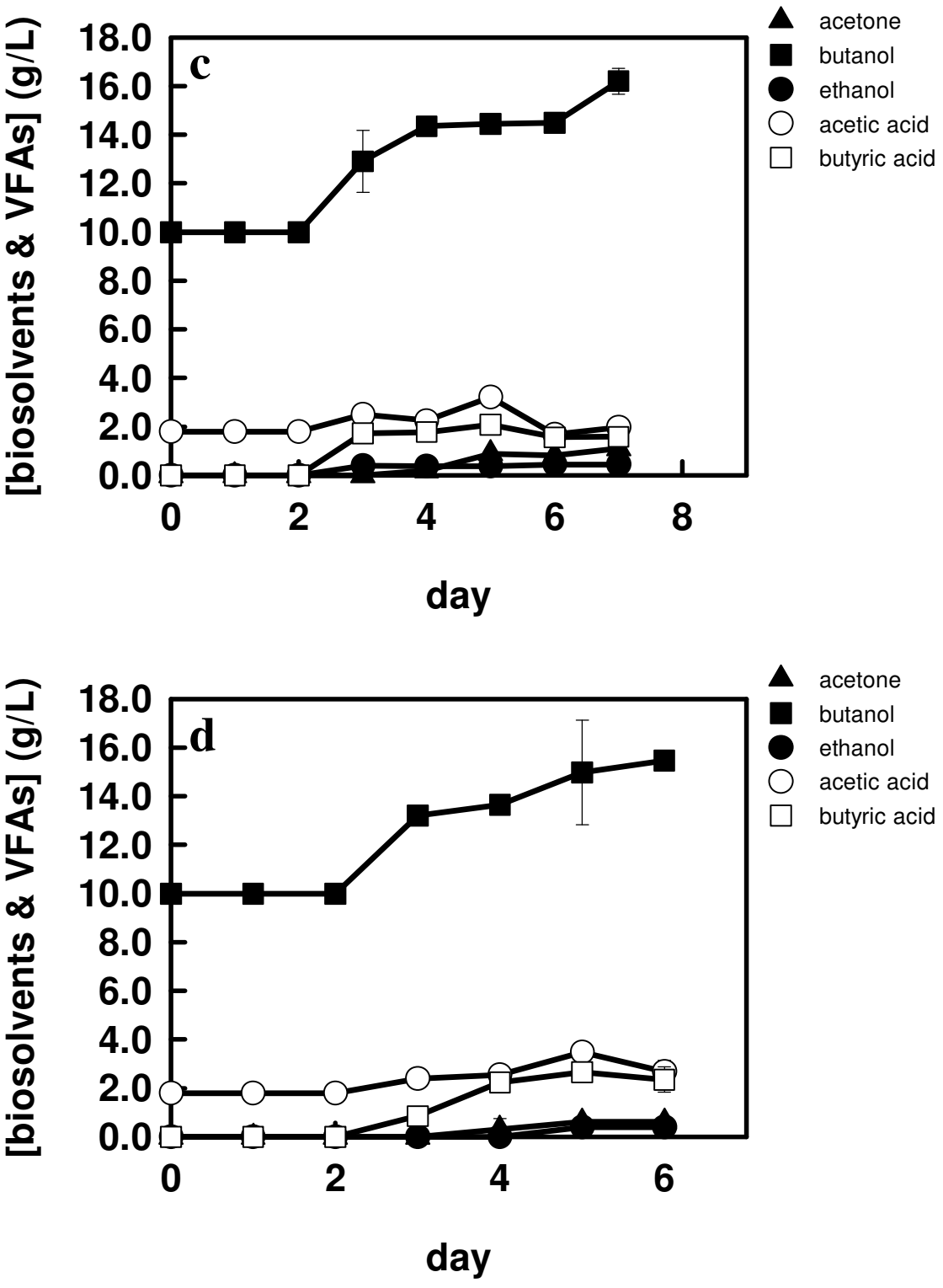


Fig 4.4. Production of biosolvents and VFAs by *Clostridium* species strain BOH3 from 30 g/L of substrate with the presence of spiked-exogenous butanol. (a) Glucose-fed, augmented-inoculation setup, 5 g/L butanol-spiking; (b) Xylose-fed, augmented-inoculation setup, 5 g/L butanol-spiking. (c) Glucose-fed, augmented-inoculation setup, 10 g/L butanol-spiking; (d) Xylose-fed, augmented-inoculation setup, 10 g/L butanol-spiking

4.4. Discussion

Augmented-inoculation setup that employed higher amount of initial cell density improved fermentation rate compared with the control. For the low concentration of substrates, i.e., 30 g/L of glucose or xylose, a significant improvement can be observed, in term of final concentration, yield, and rate of butanol production. This was observed with the higher amount of produced butanol, undetectable amount of unutilized substrates, and faster fermentation time, as shown in Fig. 4.1. This finding was in accordance with the previous studies reporting that a higher cell density to substrate ratio improves the fermentation performance (Chang et al., 1994; Berlin et al., 2006; Taherzadeh and Karimi, 2008). Various methods have been developed in previous studies, such as cell recycle and immobilization (Chang et al., 1994; Dürre et al., 2008; Lee et al., 2008), in order to improve the cell density in a bioreactor. However, solely maintaining high cell density would not assure a higher cellular productivity (Chang et al., 1994).

Further improvement was later performed to do a stepwise dosing of substrate in order to achieve an ideal fed-batch condition (Tashiro et al., 2005). Stepwise dosing of substrate resulted in a slower rate of acids production, thus cells had adequate time to re-assimilate the acids to butanol. An excessive amount of produced acids due to the acidogenic stage can be inhibitory for the cells, thus may inhibit the cell growth leading to the death phase (Lee et al., 2008).

A further improvement of augmented-inoculation setup by YEP-spiking showed an insignificant alteration to the maximum achievable butanol production. However, a shorter fermentation time was observed, in comparison with augmented-inoculation setup or its combination with stepwise dosing of substrate. Supplementation of YEP shortened the fermentation time, thus it leads to the decrease of the reactor footprint. However, since the produced butanol concentration has reached its toxicity limit, the remaining substrate was

later fermented to hydrogen and carbon dioxide. This resulted to the higher hydrogen production for the combination of stepwise dosing of substrate, augmented-inoculation setup, and YEP addition, and to the increase of total generated energy (Table 4.1 and Table 4.2). Nevertheless, the presence of complex nitrogen source YEP (as the supplements for culture growth) enables the culture to survive at high acid concentration (Lee et al., 2008). This was proven through a control experiment (data not presented) by adding exogenous acetic acid (4 g/L) and butyric acid (8 g/L) to the culture in the absence of YEP. At this condition, culture growth was inhibited and no further acidogenic or solventogenic stage occurred subsequently. This proved that the addition of YEP enhanced the culture growth and helped to diminish the inhibitory effect of high acid presences.

The natural behavior of wild-type solventogenic *Clostridia* strain BOH3 was also investigated, to assess the culture's ability in controlling pH of the fermentation broth. pH of the fermentation system was solely-governed by culture BOH3 and the absence of external pH control did not affect the fermentation performance. This result was notable for culture BOH3, as most of the butanol production studies always performed an external pH control (Fond et al., 1985; Johansson and Azar, 2007). The lowest observed pH for YEP addition setup was 4.2, while in other setups was 4.4, prior to the self-pH recovery during solventogenic stage.

The smaller reactor footprint due to faster fermentation time would reduce the capital cost, but coupled with the increase of the operation cost due to the expensive YEP addition. Inversely, combination between stepwise dosing of substrate and augmented-inoculation setup, enhanced with YEP addition, would reduce the capital cost due to shorter fermentation time, but coupled with higher operation cost. Those abovementioned conditions must be considered when the final concentration of butanol has become stagnant, as an engineering judgment for butanol production plant design. The lower overall butanol production cost as

combination between capital cost and operation cost should be determined as the most feasible option in the reactor operation mode.

Nevertheless, in comparison with other studies, the performance of culture BOH3 to convert high concentration (90 g/L) of glucose or xylose to butanol was lower than the performances of *C. acetobutylicum* 260, *C. acetobutylicum* 824, *C. acetobutylicum* 262, *C. butylicum* strain NRLL, and *C. beijerinckii BA101* (Lee et al., 2008), as shown in Table 3.1. These abovementioned cultures were able to produce comparable maximum amounts of butanol with culture BOH3, with lower concentration of glucose (~60 g/L). Overall, this study reveals an avenue to improve further the solventogenic fermentation performance of the wild-type culture BOH3, that can be replicated for butanol production at the industrial-scale, either for wild-type or genetically modified cultures.

4.5. Conclusion

In summary, higher initial cell density as implemented in augmented-inoculation setup showed an improvement in butanol and hydrogen production rate, as compared with control. A further fermentation performance improvement can be achieved by stepwise dosing of substrate and YEP addition. However, the combination of higher amount of initial cell density, substrate dosing modification, and YEP addition did not significantly increase the final achievable concentration of butanol, instead the faster fermentation rate and significant improvement of hydrogen production were observed. This was due to the shift of fermentation pathway from butanol to hydrogen production when the butanol concentration reached its toxicity limit to culture BOH3. Overall, the findings from this study provide an alternative method for the improvement of fermentation reactor operation mode in achieving a higher production rate and yield.

Table 4.1.
Kinetics of glucose fermentation in various setups

Energy-rich products	Kinetic parameters	Units	Glucose as the substrate			
			Control	AI	AI+stepwise	AI+stepwise+YEP
hydrogen	production total	(mmol)	5.400	5.145	12.108	18.444
	production rate	(mmol/day)	1.080	0.858	1.211	2.635
	production yield	(mmol/g utilized substrate)	4.853	4.505	3.363	5.123
	productivity	(mmol/g cellular protein)	1,697.73	808.78	1,903.34	2,899.34
	specific productivity rate	(mmol/g cellular protein/day)	339.55	134.80	190.34	414.19
acetone	production total	(g/L)	2.655	4.682	6.920	9.228
	production rate	(g /L/day)	0.531	0.780	0.692	1.318
	production yield	(g/g utilized substrate)	0.095	0.156	0.077	0.103
	productivity	(g/g cellular protein)	33.39	29.44	43.52	58.03
	specific productivity rate	(g/g cellular protein/day)	6.68	4.90	4.35	8.29
butanol	production total	(g/L)	4.676	7.053	13.700	13.494
	production rate	(g /L/day)	0.935	1.176	1.370	1.928
	production yield	(g/g utilized substrate)	0.168	0.235	0.152	0.150
	productivity	(g/g cellular protein)	58.80	44.35	86.14	84.84
	specific productivity rate	(g/g cellular protein/day)	11.76	7.39	8.61	12.12
ethanol	production total	(g/L)	0.360	0.380	0.843	0.912
	production rate	(g /L/day)	0.072	0.063	0.084	0.130
	production yield	(g/g utilized substrate)	0.013	0.013	0.009	0.010
	productivity	(g/g cellular protein)	4.53	2.39	5.30	5.74
	specific productivity rate	(g/g cellular protein/day)	0.91	0.40	0.53	0.82
generated energy	production total	(Joule)	10,930	16,352	29,908	33,874
	production rate	(Joule/day)	2,186	2,725	2,991	4,839
	production yield	(Joule/g utilized substrate)	9,822	13,627	8,308	9,409
	productivity	(Joule/g cellular protein)	3,436,472	2,570,491	4,701,514	5,324,863
	specific productivity rate	(Joule/g cellular protein/day)	687,295	428,412	470,155	760,697

Table 4.2.
Kinetics of xylose fermentation in various setups

Energy-rich products	Kkinetic parameters	Units	Xylose as the substrate			
			Control	AI	AI+stepwise	AI+stepwise+YEP
hydrogen	production total	(mmol)	4.22	4.03	8.99	16.16
	production rate	(mmol/day)	0.35	0.44	0.89	1.46
	production yield	(mmol/g utilized substrate)	3.51	3.36	2.49	4.48
	productivity	(mmol/g cellular protein)	1,326.74	634.94	1,413.83	2,540.31
	specific productivity rate	(mmol/g cellular protein/day)	110.56	70.55	128.53	230.93
acetone	production total	(g/L)	2.88	4.12	6.77	7.45
	production rate	(g /L/day)	0.24	0.45	0.67	0.67
	production yield	(g/g utilized substrate)	0.10	0.13	0.07	0.08
	productivity	(g/g cellular protein)	36.23	25.94	42.57	46.85
	specific productivity rate	(g/g cellular protein/day)	3.02	2.88	3.87	4.26
butanol	production total	(g/L)	4.63	7.41	10.02	11.56
	production rate	(g /L/day)	0.38	0.82	1.00	1.051
	production yield	(g/g utilized substrate)	0.16	0.24	0.11	0.12
	productivity	(g/g cellular protein)	58.24	46.62	63.05	72.70
	specific productivity rate	(g/g cellular protein/day)	4.85	5.18	5.73	6.61
ethanol	production total	(g/L)	0.61	0.90	1.22	1.72
	production rate	(g /L/day)	0.05	0.10	0.12	0.15
	production yield	(g/g utilized substrate)	0.02	0.03	0.01	0.01
	productivity	(g/g cellular protein)	7.70	5.72	7.72	10.83
	specific productivity rate	(g/g cellular protein/day)	0.64	0.64	0.70	0.99
generated energy	production total	(Joule)	11,137	16,538	24,575	29,676
	production rate	(Joule/day)	928	1,838	2,458	2,698
	production yield	(Joule/g utilized substrate)	3,094	4,594	6,827	8,243
	productivity	(Joule/g cellular protein)	3,501,566	2,599,679	3,863,181	4,664,920
	specific productivity rate	(Joule/g cellular protein/day)	291,797	288,854	351,194	424,088

CHAPTER 5

Direct conversion of food waste to butanol

Abstract

Promising results from the direct conversion of food waste to butanol has revealed the potential of food waste as an untapped energy resource that does not compete with the food supply chain. Culture BOH3 converted 210 g/L of food waste (by dry weight basis) to 9.86 g/L, 14.90 g/L, 1.94 g/L, and 15.08 mmol of acetone, butanol, ethanol, and hydrogen, within 6 days in the absence of exogenous hydrolytic enzymes. Further process improvement by increasing the amount of initial cell inoculation, doubled augmented-inoculation and tripled augmented-inoculation setups, has shifted the fermentation pathway from butanol to hydrogen production, thus increased the total cumulative hydrogen production by 19.65% and 34.48%, when compared with the control. The stagnant improvement of butanol concentration was due to the butanol toxicity limit, thus increased the hydrogen production, despite of the higher cell density application. In terms of direct conversion process, this is the first report for a direct bioconversion of food waste to butanol in the absence of separate delignification and enzymatic hydrolysis processes.

Keywords: Augmented-inoculation setup; Butanol; *Clostridium*; Direct conversion; Food waste

5.1. Introduction

Various modifications of process and reactor operation mode have been explored to improve the fermentative butanol production. Beside the process optimization, substrates selection also contributes to the fermentation performance that must be suitable for the designed process. Direct conversion as a cutting-edge process for biofuels production (Yang et al., 2009) offers a combined process chain, simplified the operation, milder process condition, lower process cost, and more environmental friendly (Lynd et al., 2002; Yang et al., 2009). Therefore, the compatibility of the selected substrates must fit with the direct conversion process. The cost of the substrate predominates the butanol production cost in a significant portion (~56%) (Qureshi and Blaschek, 2000b). Thus, the selection of a cheap and abundant substrate can significantly reduce the butanol production cost. From the process perspective, direct conversion process overcomes the high energy consumption for physical, chemical, thermal, or the combination methods, as the common delignification process (Ezeji and Blaschek, 2008). Costly commercial crude enzymes as exogenous enzymes can also be further avoided in the direct conversion process, thus decreases the overall of butanol production cost (Qureshi and Blaschek, 2000b; Mielenz, 2001; Himmel et al., 2007; Zhang et al., 2007). As an example of cellulosic ethanol, cellulase as the hydrolytic enzyme can cost ~US$ 0.30-1.00/kg of produced cellulosic ethanol (Sathitsuksanoh et al., 2010).

In comparison with direct conversion process, most of the previous studies focused on the solventogenic fermentation of hydrolysate of lignocellulosic or starchy biomass by various strains of *C. beijerinckii* or *C. acetobutylicum*, such as corn steep hydrolysate (Parekh et al., 1999), cassava, sweet potato, corn, and molasses hydrolysates (Ni and Sun, 2009), fiber-rich residue hydrolysate (Ezeji et al., 2007a),

and starch-based waste packing peanuts hydrolysate (Jesse et al., 2002). Some direct conversions from starchy biomass have also been reported, such as potato starch (Grobben et al., 1993; Gutierrez et al., 1998), cassava (Thang et al., 2010), sago, corn, and tapioca (Madihah et al., 2001). However, some of the abovementioned starchy biomasses are also food sources in many tropical countries. Thus, these starchy biomasses usage as substrates for butanol production might compete with the food supply chain (Cock, 1982; Pimentel et al., 1997; Lobell et al., 2008). These facts have strengthened the search for more suitable starchy materials, which are abundant and do not compete with the food supply chain. One such example is food waste. Food waste as the substrate for a direct conversion to butanol has never been reported previously and considered suitable, due to: (i) its non-competitive stand with food sources. (ii) its characteristic as starchy waste material that can be easily fermented through a direct conversion process. Energy can be produced by employing *Clostridium* bacteria as the most common butanol-producing bacteria to convert food waste to butanol. This hypothesis was supported by a previous study about the ability of *Clostridium* bacteria to produce amylase, pullulanase, and glucoamylase enzymes, and to perform hydrolysis process from starchy biomass (Jones and Keis, 1995).

Using food waste as a source of energy is also considered to be beneficial from a municipal solid waste management perspective. For instance in Singapore, the annual generated food waste was around 570,000 tonnes or 21.62% from the total municipal solid waste stream and treated using high-energy demand incineration process (Khoo et al., 2010). Thus, the potential of food waste as a resource of energy can be further explored, instead of treating food waste by incineration process as high energy-consuming process.

5.2. Materials and Methods

5.2.1. Food waste and defined medium preparation

Food waste was collected from a centralized bin in a canteen in the Faculty of Engineering at the National University of Singapore. Initially, food waste was sorted to exclude out the inorganic and chunky waste, such as rubber band, plastic wrapper, paper, bones, seed, and shrimp shell. Chopping and mashing processes by a food grinder was performed in order to achieve a relatively homogenous cutting size (~1.50 mm). Food waste was dried at 105 ^{o}C for 2 hours to evaporate the moisture content, thus later all of the food waste weight was based on the dry weight basis, in order to maintain the consistency of the experiment (APHA, 2005; Kaur et al., 2007). The volatile solid (VS) content was determined by measurement of sample's weight loss after 20-30 minutes heating at 550 ^{o}C (APHA 2005; Kaur, 2007).

All chemicals were purchased from Sigma Aldrich unless stated otherwise. Dried food waste with various concentrations was prepared in the 60-mL bottles with the reduced mineral salts medium. The reduced mineral salts medium was prepared (He et al., 2003), supplemented with Wolin solution (Wolin et al., 1963), and initial pH adjustment from neutral to 5.0–5.2 by adding concentrated H_2SO_4 under continuous N_2 purging. The subsequent experiments were conducted in 60-mL bottles containing 36-mL of the pH-adjusted medium.

5.2.2. Microorganism and culture activation

Laboratory stock of *Clostridium butyricum* strain BOH3 was activated at 35 ^{o}C in a 125-rpm shaking incubator for 48 hours, prior to the fermentation experiment. The activation medium was 36-mL of mineral salt medium that spiked with 10 g/L of glucose in 60-mL bottles with 10% culture inoculation (v/v). After 48 hours of activation, the 4-mL of active culture BOH3 was centrifuged at 10,000 rpm for 10

minutes to concentrate the cell. The supernatant was carefully decanted in the anaerobic chamber, followed by the addition of 4-mL of sterilized mineral salts medium to re-dissolve the concentrated cell pellet, prior to the inoculation into a new bottle containing 36-mL of defined medium. This inoculation method is termed as singled augmented-inoculation setup or control. Separation of supernatant and pellet was aimed to minimize the effects of residual monosaccharides and volatile fatty acids (VFAs) from the activation medium, as well as increasing the initial amount of cell.

In the further enhancement process experiments, a two- and three-fold amount of cells as compared with control setup were implemented for inoculation. This was performed while maintaining the volume of the inoculums of 4-mL of active cells in mineral salt medium that was spiked with 10 g/L of glucose, respectively. These latter two methods are termed as doubled and tripled augmented-inoculation setups.

5.2.3. Optimization of batch fermentation and inhibition study

Triplicates of 1.2-9.6 g of food waste (by dry weight basis) was added into 36-mL of mineral salt medium in 60-mL bottles. With the addition of 4-mL of active cultures, the total volume of 40-mL was achieved and made the final concentrations of food waste in the range of 30-240 g/L. From the various concentrations of food waste, the most optimum food waste concentration was determined from the highest final concentration, rate, yield, and productivity of acetone, butanol, ethanol, and hydrogen. The determined food waste concentration was implemented in subsequent experiments. The experiment was further optimized by increasing the initial amount of cell density (doubled and tripled augmented-inoculation setups), in comparison with control.

Similarly, the most optimum augmented-inoculation setup was later determined with the assessment of butanol toxicity to the culture. A study was conducted by addition of exogenous 4.5, 9.0, and 13.5 g/L of butanol at the initial stage of fermentation with the aforementioned food waste concentration and augmented-inoculation setup. The experimental sampling and analyses were conducted daily for gaseous and liquid samplings.

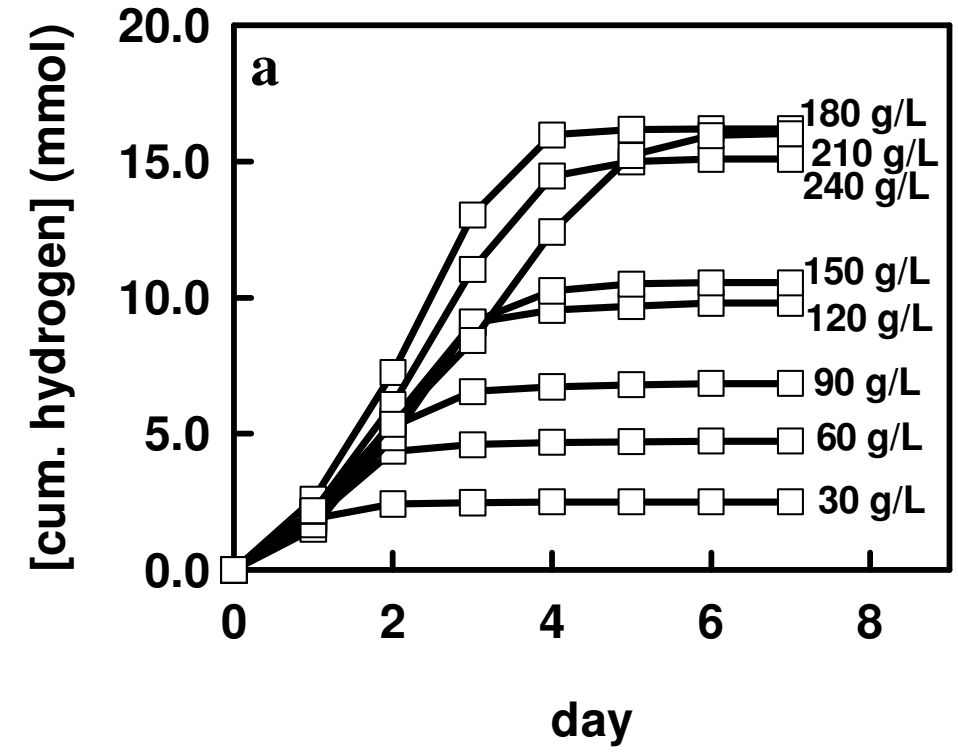
5.2.4. Chemical analysis

Refer to subchapter 3.2.3.

5.3. Results

5.3.1. Optimization of food waste concentration

Various concentrations of food waste, i.e., 30, 60, 90, 120, 150, 180, 210, and 240 g/L were applied in direct conversion experiments. Control condition was performed with 0.07 g cellular protein/L as the initial amount of cell inoculum. Results showed that the hydrogen, acetone, butanol, and ethanol concentrations were coupled with the increase of food waste concentrations (Fig 5.1).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10.0
5.0
0.0
0
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4
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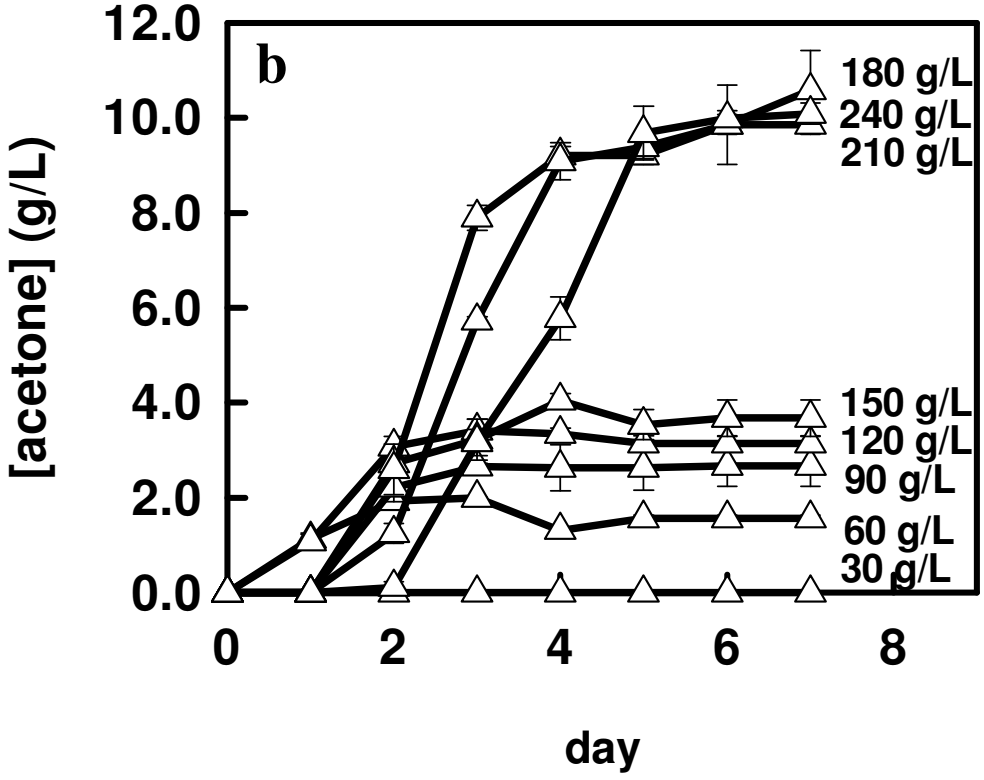
30 g/L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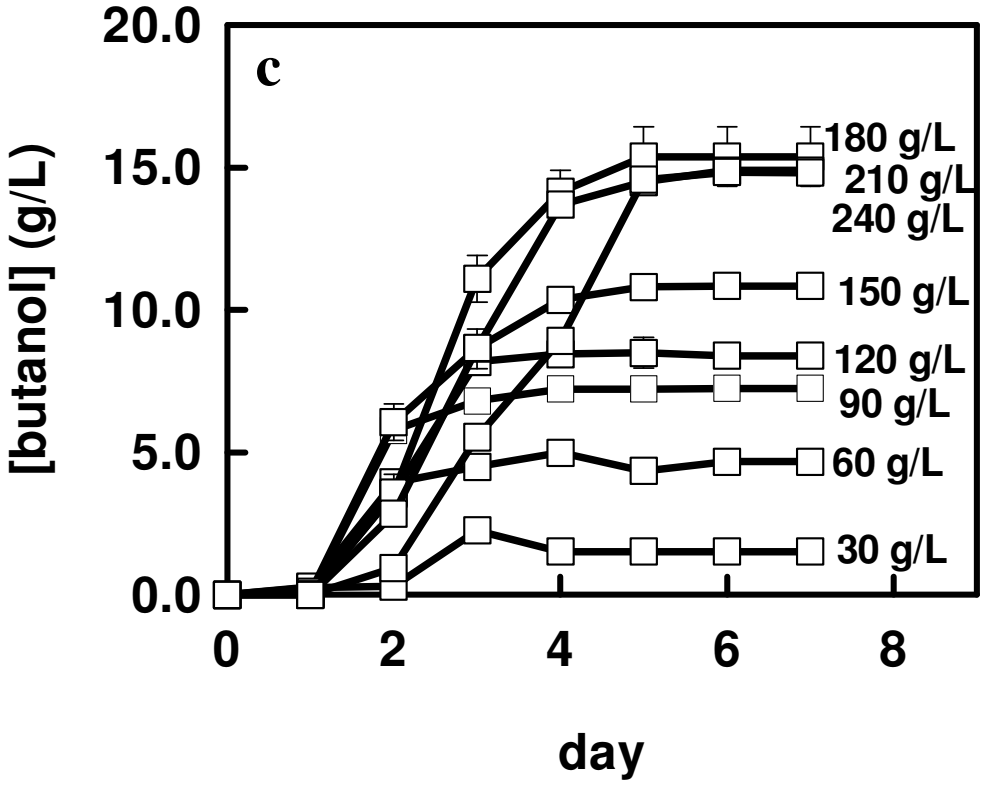


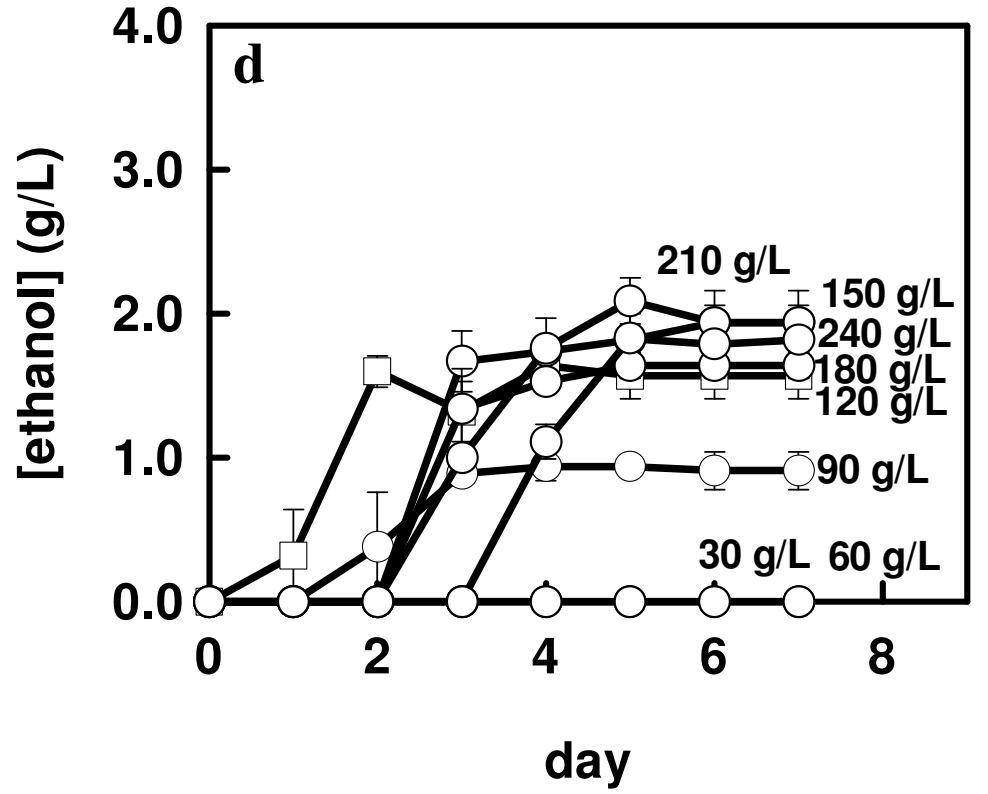


Fig. 5.1. Conversion profiles from various food waste concentrations: (a) hydrogen; (b) acetone; (c) butanol; and (d) ethanol

The highest produced cumulative hydrogen was 16.02 mmol, which was produced from 180 g/L of food waste. The highest produced acetone was 10.09 mmol from 240 g/L of food waste. The highest produced butanol and ethanol concentrations were 14.90 g/L and 1.94 g/L, respectively, both from 210 g/L of food waste. The 240 g/L of food waste was also the highest energy generation that produced 37,708 J. From other kinetic parameters, such as rate and productivity, most of the best conditions fell on 180 g/L of food waste. Hence, the optimum condition was chosen as the median between 180 g/L and 240 g/L of food waste.

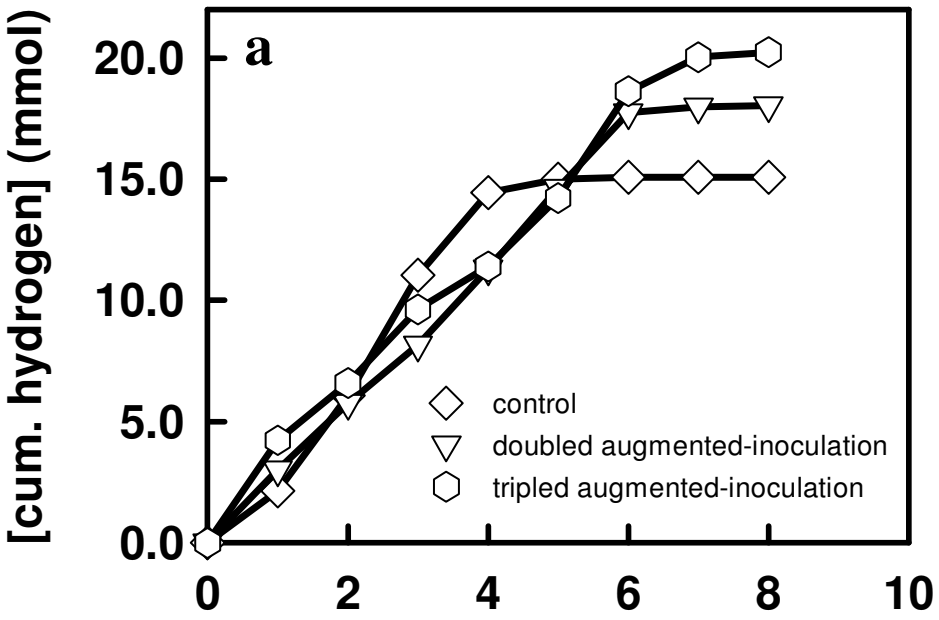
Glucose and xylose as hydrolysis products were also detected prior and after the conversion processes. At the pre-conversion process, hydrolysis of food waste occurred due to the high temperature and pressure during the autoclaving process, in which 0.98 g/L and 0.76 g/L of glucose and xylose were detected in all food waste concentrations. For the 210 g/L of food waste as the substrate, 24.3 g/L of glucose and 20.5 g/L of xylose were detected in the end of the conversion.

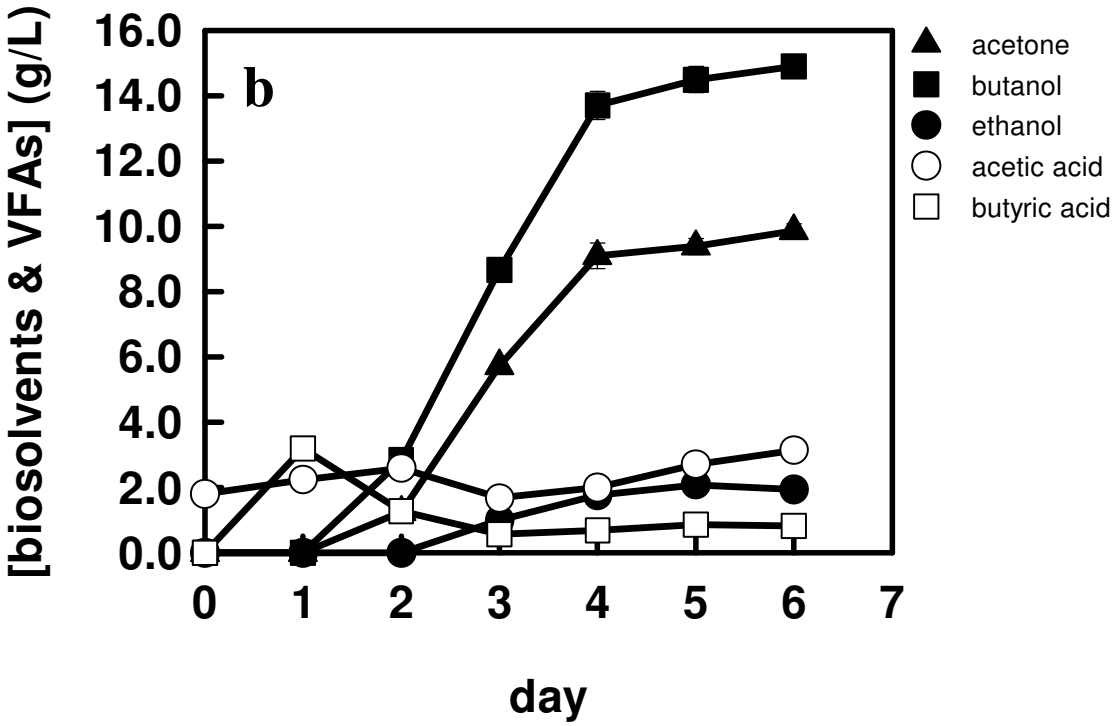
Final cell density was 0.06 g cellular protein/L that was lower than the initial amount 0.07 g cellular protein/L. The decrease of cell concentration is commonly found in the end of solventogenic fermentation due to the presence of butanol as toxic product for cell. In spite of the exponential cell growth occurred in the acidogenic stage, the depletion of cells was still observed during the solventogenic stage (Fond et al., 1986).

5.3.2. Optimization of the augmented-inoculation setup

From the food waste concentration optimization experiments, it was concluded that 210 g/L was the most optimum food waste concentration for the overall conversion process. Doubled and tripled augmented-inoculation setups were implemented, to further increase the conversion efficiency, by applying initial cell

density inoculum of 0.14 g cellular protein/L and 0.21 g cellular protein/L respectively. However, butanol production performances did not significantly improve, instead hydrogen production hiked (Fig 5.2).





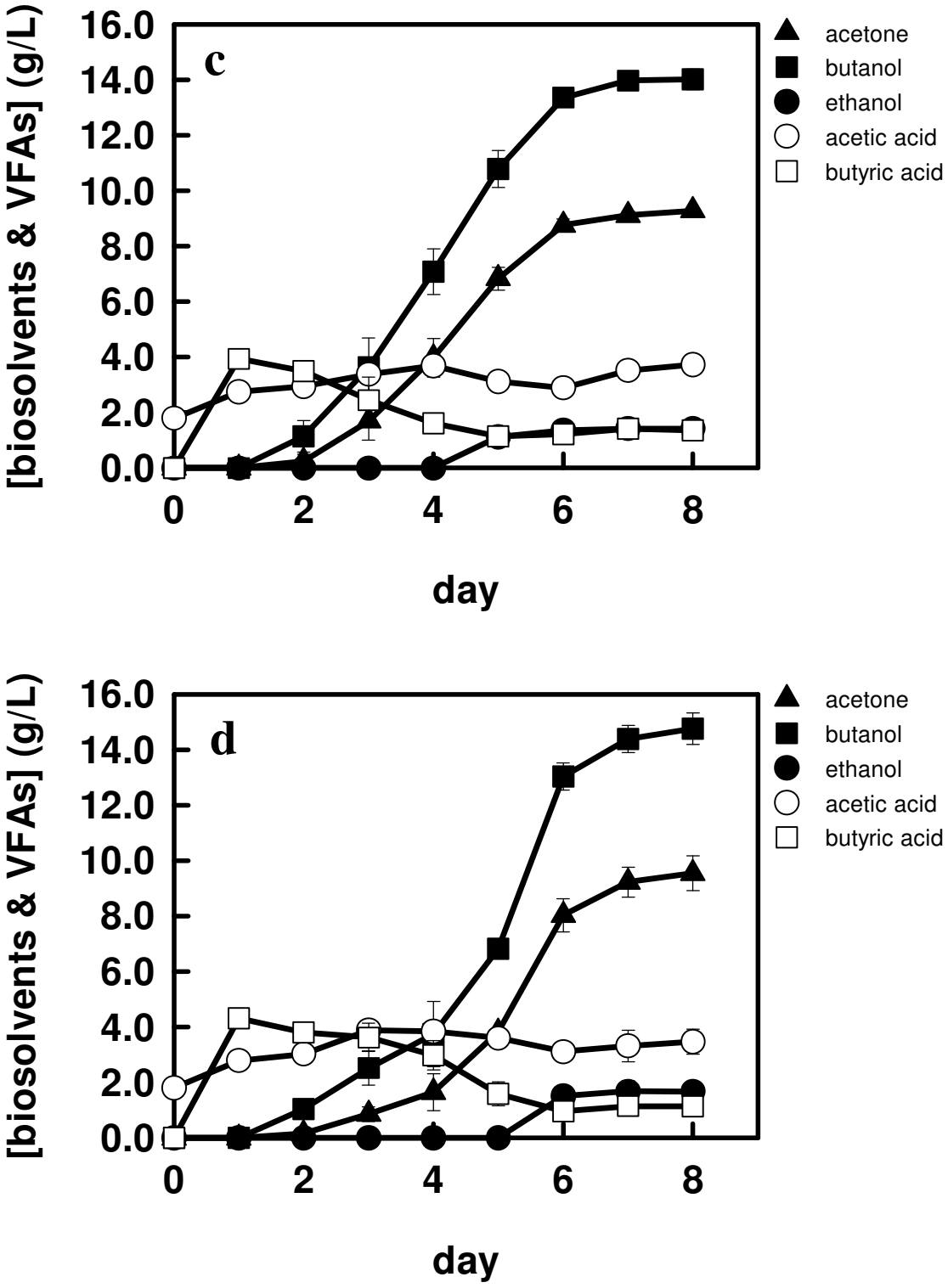


Fig. 5.2. Conversion profiles from 210 g/L of food waste with augmented-inoculation setup: (a) hydrogen production for control, doubled augmented-inoculation, and tripled augmented-inoculation setups; (b) singled augmented-inoculation setup as control; (c) doubled augmented-inoculation setup; (d) tripled augmented-inoculation setup

The incremental initial amount of cell inoculum was coupled with the improvement of hydrogen production, but it did not show a significant improvement for acetone, butanol, and ethanol productions. Hydrogen production has increased from 15.08 mmol for the control to 18.04 mmol and 20.24 mmol for the doubled and tripled augmented-inoculation setups, respectively. The butanol concentrations were comparably similar from 14.90 g/L with control to 14.03 g/L and 14.76 g/L of the doubled and tripled augmented-inoculation setups. Lag phase was not observed for the doubled and tripled augmented-inoculation setups, however, the fermentation ceased in prolonged times, which were 6 days for the control and 8 days for the other both setups. The higher amount of acids was observed in the tripled augmented-inoculation compared with the control and doubled augmented-inoculation setup, which were 7.10 g/L, 5.44 g/L, and 6.69 g/L within 24 hours of fermentation, respectively. The lowest observed pH was 4.4 for the doubled and tripled augmented-inoculation setups, while 4.6 was the lowest one for the control.

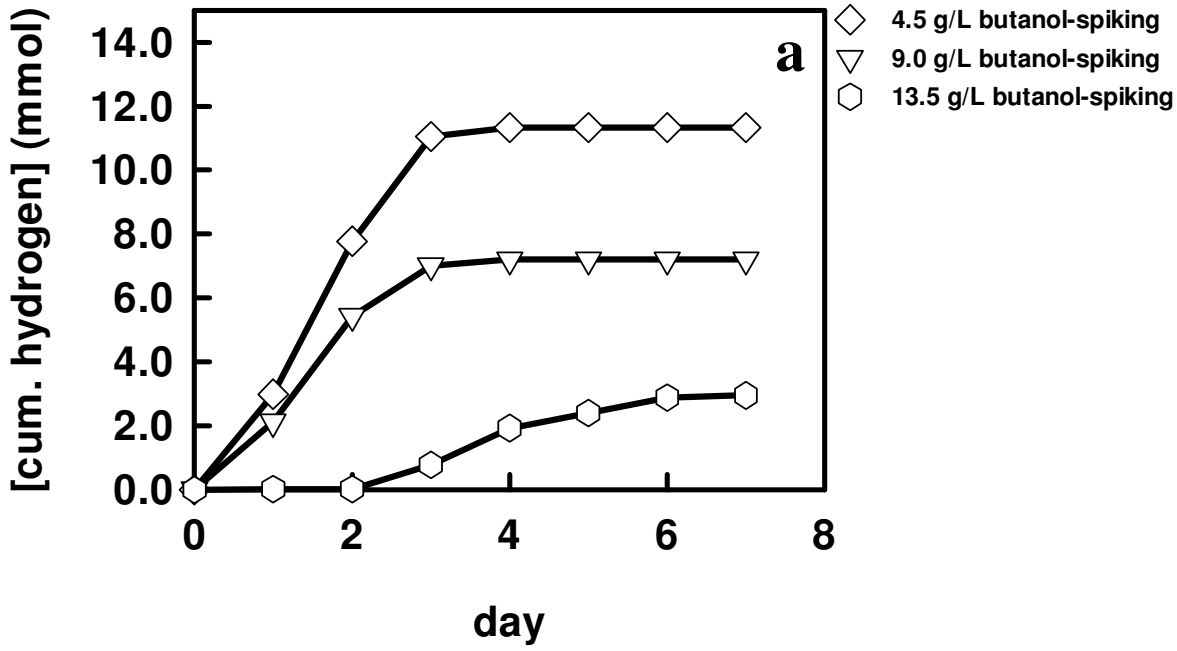
Nevertheless, the control showed the highest butanol production, and the hydrogen production was the lowest in comparison with doubled and tripled augmented-inoculation setups. The improvements of hydrogen production compared to control were 19.65% and 34.48%. This result of the tripled augmented-inoculation setup showed a better overall conversion performance compared to other setups. Thus, tripled augmented-inoculation setup was later used for the subsequent experiments.

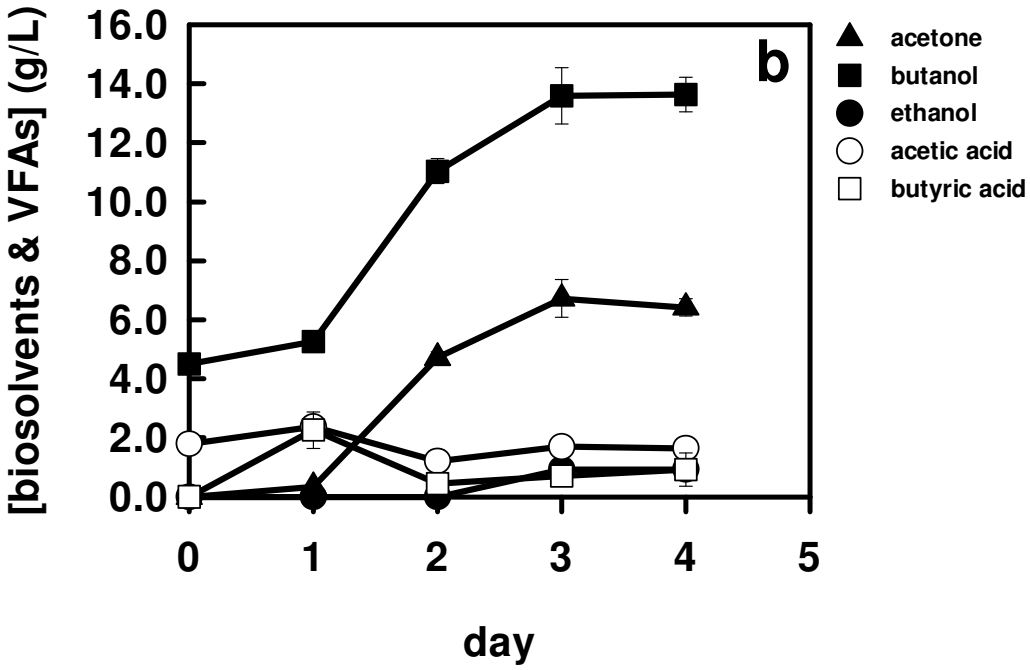
The decrease of cell density at the end of fermentation was observed in the control, doubled, and tripled augmented-inoculation setups. This common phenomenon in the solventogenic performance was observed for culture BOH3, which could be due to the substrate transfer limitation (Tashiro et al., 2005) and the

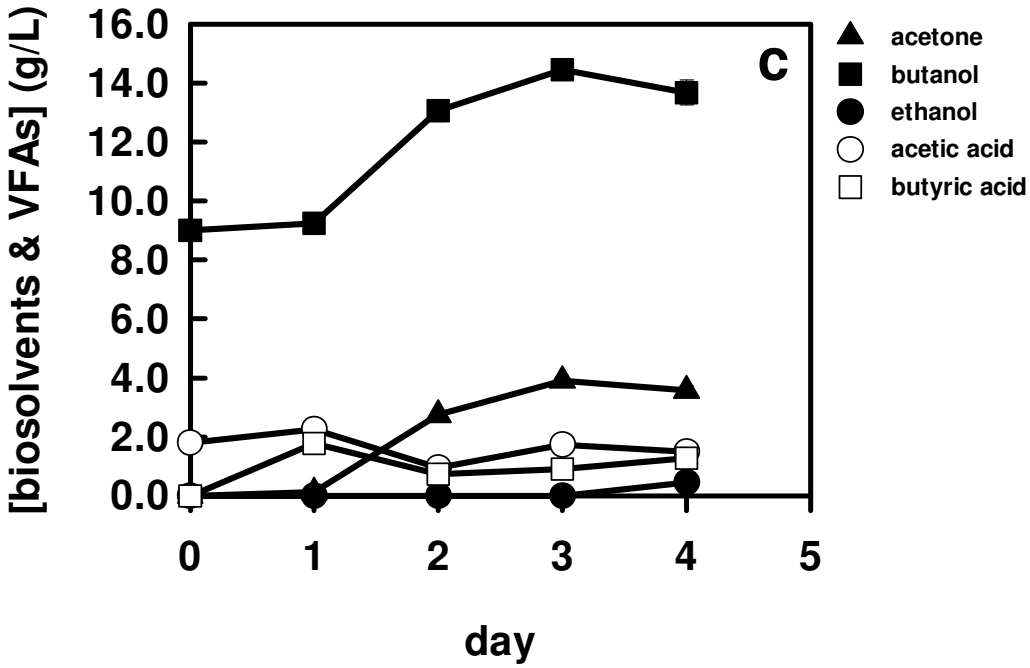
presence of toxic butanol (Lee et al., 2008). The substrate transfer limits the incremental of cell density, thus cell bleeding is recommended in order to maintain the maximum concentration of cells in the reactor (Tashiro et al., 2005). For the control, doubled, and tripled augmented-inoculation setups, the final amount of cell density in term of cellular protein were 0.06 g/L, 0.09 g/L, and 0.13 g/L, while the initial concentrations were 0.07 g/L, 0.14 g/L, and 0.21 g/L, respectively.

5.3.3. Determination of butanol toxicity level

Isolate BOH3 has never reached butanol concentration above 15 g/L for batch bottles experiments. This was affected by the butanol property that inhibits the cell growth up to 50% at the butanol concentrations of 7.4-11.1 g/L (Lee et al., 2008). In order to further confirm that the toxicity of butanol as the reason for the plateu amount of produced butanol, spikes of exogenous 4.5 g/L, 9.0 g/L, 13.5 g/L, and 20 g/L of butanol were conducted with food waste as rge substrate. Experiments were performed with the tripled augmented-inoculation setup as the chosen optimum augmented-inoculation setup (Fig 5.3).




b
[biosolvents & VFAs] (g/L)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
1
2
3
4
5
day
acetone
butanol
ethanol
acetic acid
butyric acid


c
[biosolvents & VFAs] (g/L)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
1
2
3
4
5
day
acetone
butanol
ethanol
acetic acid
butyric acid

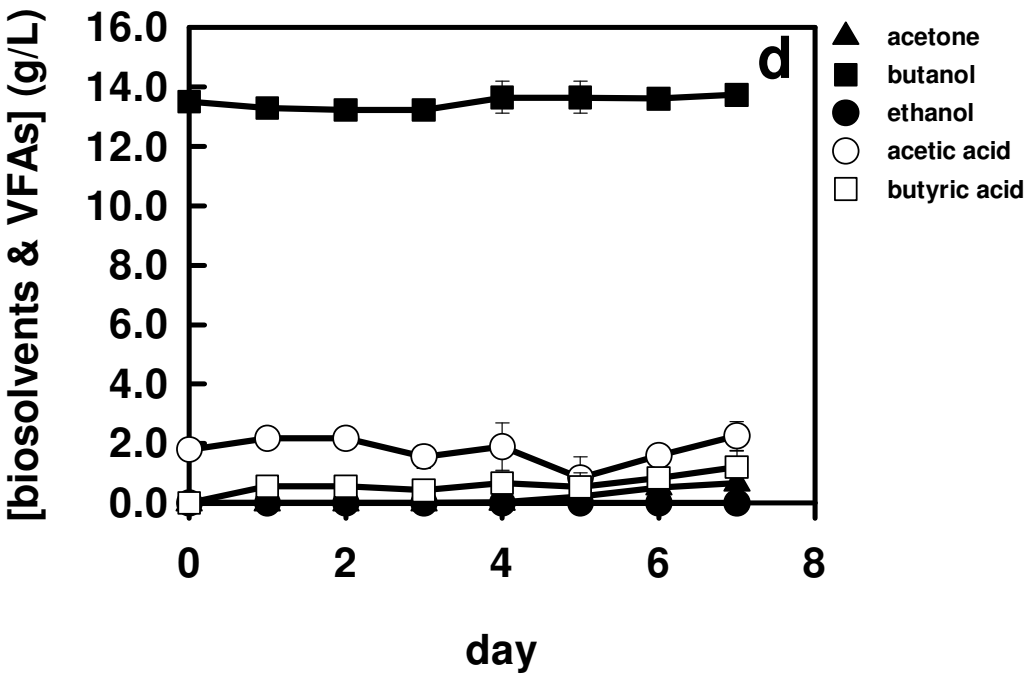


Fig. 5.3. Conversion profile from 210 g/L of food waste with a tripled augmented-inoculation setup: (a) hydrogen production; (b) 4.5 g/L butanol-spiking; (c) 9.0 g/L butanol-spiking; (d) 13.5 g/L butanol-spiking

In the presence of 4.5, 9.0, and 13.5 g/L of butanol, the culture was able to produce 9.13 g/L, 4.68 g/L, and 0.23 g/L of butanol. However, no culture growth or butanol production was observed when the culture was spiked with 20 g/L of butanol. Production of hydrogen decoupled with the incremental of spiked exogenous butanol concentrations, which were 11.33 mmol, 7.20 mmol, and 2.95 mmol, respectively. This showed the culture's ability to convert food waste, despite of the presence of high concentration of butanol.

The final amounts of cell density were similar with 0.06 g cellular protein/L for all of the spiked-butanol concentrations. Thus, high butanol concentration, either from the produced or spiked butanol, affected the cell activity. Glucose and xylose were also detected with butanol spiking. 25.90 g/L and 22.10 g/L of glucose and

xylose were detected in the 4.5 g/L butanol-spiking setup, 24.90 g/L and 21.10 g/L of glucose and xylose in the 9.0 g/L butanol-spiking setup, while 17.60 g/L and 13.90 g/L of glucose and xylose in the 13.5 g/L butanol-spiking setup.

5.4. Discussion

Preliminary experiments was conducted to improve butanol production through butyric acid addition and heat shock treatment with a fixed concentration of food waste via a direct conversion process. However, no improvements were observed under both conditions (data not showed). With the fixed amount of fermentable substrate that contained in the food waste, the sole process parameter in direct conversion process that can be adjusted was by increasing the food waste concentration. However, this process adjustment was in the expense of the high amount of unutilized food waste in the end of conversion. This was due to the inaccessibility of culture BOH3 to fully-utilize all fermentable substrates in the food waste to produce butanol. The unutilized food waste later can be further converted to other value-added products, such as compost, due to the availability of Volatile Solid (VS) as the remnant.

For calculation of yield, some denominators can be selected as by dry weight basis, such as the total amount of substrate, total carbon, or total VS. Total VS was selected as the most appropriate parameter to assess the yield compared with the other two denominators, as the presence of non-carbonaceous compounds (such as proteins, vitamins, fat, etc.) in the food waste influenced the butanol production. Moisture and VS contents of the food waste in this experiment were 75.56% and 92.71% (by dry weight basis), respectively.

Consideration of 210 g/L as the most optimum concentration for a direct conversion was based on several parameters, such as the final concentration, total generated energy, yields, rates, and productivities of the produced acetone, butanol, ethanol, and hydrogen as energy-rich products. The summarized conversion performances from various food waste concentrations are presented (Table 5.1).

As shown in Table 5.1., 180 g/L of food waste showed the superiority in most of the productions, yields, and rates for hydrogen. However, 240 g/L showed the highest acetone production and generated energy. Despite 210 g/L of food waste was used as the baseline in the subsequent experiments, the performance discrepancy between 180 g/L and 240 g/L of food waste might be due to the average and standard deviation values of the measurements. The food waste that originated from similar source was homogenized prior to the conversion.

In particular, for 210 g/L of food waste, the yields for acetone, butanol, and ethanol were 5%, 8%, and 1% from the available VS or the total of 14% from available VS has been converted to biosolvents. 15.08 mmol of hydrogen was produced from 210 g/L of food waste, thus gave a yield of 0.38 % from the available VS. The remaining VS was converted to cells, carbon dioxide, acetic acid, butyric acid, glucose, xylose, and as remnant of unutilized food waste. In comparison with glucose as the substrate, previous study showed that isolate BOH3 was able to produce comparably similar amount of biosolvents from 90 g/L of glucose (Fig. 4.2.). 210 g/L of food waste with 92.71% VS content (by dry weight basis) had the equal performance with 90 g/L VS of glucose. Thus, the performance of direct conversion of food waste to butanol by culture BOH3 was 46.22%.

The lower performance of direct fermentation was expected due to the hydrolysis of food waste as the rate limiting step that inhibited the culture to access

and convert all VS to butanol or other conversion products. Enzymatic hydrolysis process is expected to depolymerize the polysaccharides in the food waste, thus the free monosachharides can be produced in higher amount (Lynd et al, 2002). The higher amount of monossacharides would produce higher amount of butanol, but limited to the butanol toxicity. Furthermore, during the collection of food waste, it was observed that the presence of unknown substances in the food waste (such as, washing detergent, oil, grease, food taste enhancer, food preservatives, etc.) might inhibit the culture growth. Therefore, the presence of various substances in the food waste accommodated the substrates competition for the culture (Farhat et al., 1998; Thassitou and Arvanitoyannis, 2001).

The amount of produced butanol in this direct conversion process was capped ~15 g/L, even from 240 g/L of food waste. This was limited by the toxicity of butanol as product inhibition to the solventogenic *Clostridia* that started to be inhibitive at ~7.4-11.1 g/L of butanol (Lee et al., 2008). An investigation using glucose or xylose also showed the butanol toxicity that has never achieved > 15 g/L, as shown in Fig 4.2. Thus, butanol concentration above 15 g/L was considered to completely inactivate the cell of culture BOH3. Free glucose and xylose as hydrolysis products were also detected in the end of conversion, but the culture was unable to utilize those abovementioned monosaccharides due to the presence of toxic butanol. From carbon balance standpoint, 44.33% of carbon was not utilized in the fermentation of 210 g/L of food waste. This demonstrated the inability of the culture to further hydrolyze and ferment the unutilized food waste.

Table 5.1.
Conversion kinetics from various food waste concentrations

Energy-rich products	Kinetic parameters	Units	Food waste concentrations g/L (by dry weight basis)							
			30	**60**	**90**	**120**	**150**	**180**	**210**	**240**
hydrogen	production total	(mmol)	2.50	4.73	6.85	9.79	10.54	16.20	15.08	16.02
	production rate	(mmol/day)	0.62	0.79	1.14	1.63	1.76	3.77	2.76	2.60
	production yield	(mmol/g utilized substrate)	2.24	2.13	2.05	2.20	1.90	2.43	1.94	1.80
	productivity	(mmol/g cellular protein)	41.38	78.42	113.58	162.26	174.77	268.53	249.99	265.64
	specific productivity rate	(mmol/g cellular protein/day)	10.35	13.07	18.93	27.04	29.13	62.57	45.80	43.08
acetone	production total	(g/L)	0.00	1.57	2.67	3.14	3.68	9.21	9.86	10.09
	production rate	(g/L/day)	0.00	0.26	0.45	0.52	0.61	2.15	1.81	1.64
	production yield	(g/g utilized substrate)	0.00	0.03	0.03	0.03	0.03	0.06	0.05	0.05
	productivity	(g/g cellular protein)	0.00	26.08	44.32	52.04	60.94	152.62	163.47	167.20
	specific productivity rate	(g/g cellular protein/day)	0.00	4.35	7.39	8.67	10.16	35.56	29.95	27.11
butanol	production total	(g/L)	1.52	4.67	7.24	8.39	10.85	14.09	14.90	14.82
	production rate	(g/L/day)	0.38	0.78	1.21	1.40	1.81	3.28	2.73	2.40
	production yield	(g/g utilized substrate)	0.05	0.08	0.09	0.08	0.08	0.08	0.08	0.07
	productivity	(g/g cellular protein)	25.13	77.46	120.06	139.06	179.90	233.51	247.07	245.75
	specific productivity rate	(g/g cellular protein/day)	6.28	12.91	20.01	23.18	29.98	54.41	45.26	39.85
ethanol	production total	(g/L)	0.00	0.00	0.91	1.57	1.94	1.64	1.94	1.82
	production rate	(g/L/day)	0.00	0.00	0.15	0.26	0.32	0.38	0.35	0.29
	production yield	(g/g utilized substrate)	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
	productivity	(g/g cellular protein)	0.00	0.00	2.52	4.34	5.37	6.33	5.88	4.89
	specific productivity rate	(g/g cellular protein/day)	0.00	0.00	0.42	0.72	0.90	1.47	1.08	0.79
generated energy	production total	(Joule)	2,605	9,114	15,333	18,846	23,333	34,931	36,836	37,078
	production rate	(Joule/day)	651	1,519	2,556	3,141	3,889	8,139	6,749	6,013
	production yield	(Joule/g utilized substrate)	2,342	4,096	4,594	4,235	4,195	5,233	4,730	4,166
	productivity	(Joule/g cellular protein)	43,189	151,090	254,182	312,418	386,793	579,063	610,637	614,653
	specific productivity rate	(Joule/g cellular protein/day)	10,797	25,182	42,364	52,070	64,465	134,927	111,872	99,673

Further improvement of butanol production was performed by increasing the initial amount of cell inoculum by two and three-folds. This condition was developed to mimic a bioreactor that could retain high amount of biomass that can improve biological process performance (Chang et al., 1994; Ng and Hermanowicz, 2005). However, the production of butanol did not improve significantly in the doubled and tripled augmented-inoculation setups, even slightly decreased from 14.90 g/L for control, to 14.03 g/L and 14.76 g/L for doubled and tripled augmented-inoculation setups, respectively. This might be due to that the acidogenesis rate of culture BOH3 was higher than the solventogenesis rate or the higher rate of acids production compared to rate of acid re-assimilation to solvents, coming from higher initial amount of cell. It was observed that the lower amount of acids was produced in the control (5.44 g/L) compared with 6.69 g/L and 7.52 g/L for doubled and tripled augmented-inoculation setups. Higher acids concentration indicated the higher amount of ATP for cell growth (Kraemer and Bagley, 2007), but cells were unable to efficiently re-assimilate the acids to higher amount of solvents, including acetone and ethanol, due to the butanol toxicity. The requirement for the amount of acids by other solvents-producing isolates is around 3–4 g/L prior to the onset of solventogenic stage (Assobhei et al., 1998). However, a preliminary experiment showed culture BOH3 could sustain a much higher acids concentration prior to its onset of solventogenic, ~7 g/L (data not presented). Thus, this also revealed the high tolerance of culture BOH3 to high external acids concentration.

The higher initial amount of cell inoculum also shifted the conversion pathway to hydrogen instead of butanol, due to the presence of toxic butanol. As observed during the fermentation, the shift of fermentation pathway to butanol was due to the extended acidogenic stage that indicated by the higher amount of produced acids and

hydrogen. Higher amount of produced acids gave a lag phase to the culture in converting to butanol, concurrently higher hydrogen production would be observed. This explained the improvement of hydrogen production to 19.65% and 34.48% for doubled and tripled augmented-inoculation setups, respectively, compared with the control. The conversion performances from various augmented-inoculation setups with 210 g/L of food waste are summarized (Table 5.2).

Table 5.2.
Conversion kinetics from 210 g/L of food waste with various augmented-inoculation setups

Energy-rich products	Kinetic parameters	Units	Setups		
			Control	Doubled augmented-inoculation	Tripled augmented-inoculation
hydrogen	production total	(mmol)	15.08	18.04	20.28
	production rate	(mmol/day)	2.76	2.55	2.86
	production yield	(mmol/g utilized substrate)	1.94	2.32	2.60
	productivity	(mmol/g cellular protein)	249.99	149.56	112.06
	specific productivity rate	(mmol/g cellular protein/day)	45.80	21.11	15.82
acetone	production total	(g/L)	9.86	9.29	9.54
	production rate	(g/L/day)	1.81	1.31	1.35
	production yield	(g/g utilized substrate)	0.05	0.05	0.05
	productivity	(g/g cellular protein)	163.47	77.01	79.04
	specific productivity rate	(g/g cellular protein/day)	29.95	10.87	11.16
butanol	production total	(g/L)	14.90	14.03	14.76
	production rate	(g/L/day)	2.73	1.98	2.08
	production yield	(g/g utilized substrate)	0.08	0.07	0.08
	productivity	(g/g cellular protein)	247.07	116.27	81.54
	specific productivity rate	(g/g cellular protein/day)	45.26	16.42	11.51
ethanol	production total	(g/L)	1.94	1.42	1.67
	production rate	(g/L/day)	0.35	0.20	0.24
	production yield	(g/g utilized substrate)	0.01	0.01	0.01
	productivity	(g/g cellular protein)	5.88	1.66	1.30
	specific productivity rate	(g/g cellular protein/day)	1.08	0.23	0.18
generated energy	production total	(Joule)	36,836	35,140	37,206
	production rate	(Joule/day)	6,749	4,961	5,253
	production yield	(Joule/g utilized substrate)	4,730	4,512	4,778
	productivity	(Joule/g cellular protein)	610,637	291,261	205,592
	specific productivity rate	(Joule/g cellular protein/day)	111,872	41,119	29,025

High concentration of cells was applied for the butanol toxicity experiments, in order to protect the cells from the shear/stress environment (Chang et al., 1994). However, high cell density in the fermentation system as applied in the tripled augmented-inoculation setup also has disadvantages, such as the decrease in specific activity or viability of cells due to nutrient limitations and difficulty to maintain sterile condition (Chang et al., 1994). Thus, the plateu butanol production from the food waste was further confirmed due to the butanol toxicity, rather than the hindrance of food waste inaccessibility to the culture. This result was also in accordance with the butanol toxicity limit for this culture in the previous study, by using glucose or xylose as the substrate.

In comparison with other studies about direct conversion of starchy biomass to butanol, this study showed its avenue to enlist food waste as one of the potential substrate candidates for butanol production. The pure compounds and food staple substrate in the previous studies, such as cassava starch, corn powder, potato starch, sago starch, and tapioca powder, have put food waste as another substrate alternative that is not considered as food staple. Nevertheless, culture BOH3 showed a potential as an amylolytic culture due to its ability in utilizing food waste that contains starch or amylum. However, its performance can be further improved to enable its ability in producing high yield of butanol from food waste. This study also remarks an opportunity to solve the real situation for food waste treatment in municipal solid waste system. Furthermore, the possibility to perform an affordable butanol production from waste biomass that does not compete with the food chain supply is revealed. The comparison with the previous studies about direct conversion of starchy biomass to butanol is elaborated (Table 5.3).

Table 5.3.
Previous studies about direct conversion of starchy biomass to butanol

Substrates	Substrates concentration (g/L)	Cultures	Energy-rich substances products				References
			Acetone	Butanol	Ethanol	Hydrogen	
			(g/L)			(mmol)	
corn powder	30	*C. acetobutylicum* P262	2.91	8.61	0.36	not defined	Madihah et al., 2001
tapioca powder	30	*C. acetobutylicum* P262	1.64	4.89	0.20	not defined	Madihah et al., 2001
potato powder	30	*C. acetobutylicum* P262	1.17	3.34	0.13	not defined	Madihah et al., 2001
sago starch	60	*C. acetobutylicum* P262	1.67	16.00	0.34	not defined	Madihah et al., 2001
cassava starch	60	*C. saccharoperbutylacetonicum* N1-4	3.60	16.90	0.50	not defined	Thang et al., 2010
cassava starch	60	*C. acetobutylicum* EA2018	5.00	13.00	1.00	not defined	Gu et al., 2009
potato powder	140	*C. acetobutylicum* DSM1731	4.00	12.50	1.00	not defined	Grobben et al., 1993
food waste	**210**	***C. butyricum* BOH3**	**9.86**	**14.90**	**1.94**	**15.08**	**this study**

5.5. Conclusion

The most optimum food waste concentration for a direct conversion to butanol was 210 g/L (by dry weight basis) that produced ~15 g/L of butanol. In the presence of high amount of butanol, the toxicity limit of butanol was the rate limiting step for the process performance in the batch bottle setup. Butanol production improvement through implementation of higher amount of cell inoculum was hindered due to the butanol toxicity limit, thus shifted the fermentation pathway from butanol to hydrogen production. A significant improvement of hydrogen production was observed in the high-cell density inoculation compared to control, while butanol production started to be plateau. The high amount of unutilized food waste as the substrate was the consequence for the omission of separate enzymatic hydrolysis, which requires further stabilization process at the downstream treatment. This gives more various added-value products from the food waste residue that can be further exploited, such as compost, methane, hydrogen, etc., thus maximizes the energy generation from food waste.

CHAPTER 6

Conclusions, implications, and recommendations

6.1. Conclusions and implications

1. A wild-type isolate of bacteria that harbors solventogenic, celulolytic, and xylanolytic performances has been isolated. The culture, designated culture BOH3, was isolated from a paddy field soil. The sequence of the 16S rRNA gene amplified from the genomic DNA of culture BOH3 exhibited 98% identity to the sequence of *Clostridium butyricum* W4. The nucleotide sequence was deposited in the GenBank database with an accession number HQ830243.
2. Glucose and xylose were able to be converted to butanol in a higher yield compared with other solventogenic *Clostridia*. This culture also showed a high resistance to the presence of butanol in the fermentation broth, thus indicating the robustness of the culture.
3. As a wild-type isolate, culture BOH3 has lagging properties, such as inhibition from high concentration of substrate dosed in bulkdosing. This created an avenue to modify the substrate dosing method, namely stepwise dosing method, to dose the substrate gradually to the fermentation process in a batch mode.
4. Through the application of stepwise dosing of substrate, all of the dosed substrate can be fully-utilized by culture BOH3 and maximized the produced butanol. Coupled with the stepwise dosing of substrate, high cell density was inoculated to further enhance the fermentation performance. Results showed that the aforementioned combination improved the butanol production rate, even with the absence of yeast extract-peptone (YEP) addition. This finding emerges another

research hiatus for the application of such dosing method for genetically-modified cultures, as wild-type cultures (culture BOH3 as a model) performed better with this substrate feeding method.

5. YEP addition fastened the fermentation time, but did not alter the maximum achievable butanol concentration. Thus, the final concentration of butanol was not improved, instead of improved butanol production rate. A fermentation shift from butanol to hydrogen production pathway was also observed, when the toxicity limit of butanol has been achieved. This resulted in the significant production of hydrogen and the incremental of the total generated energy from the fermentation process. However, YEP omission was permittable when the fermentation system employed the stepwise dosing of substrate and augmented-inoculation setup.
6. Culture BOH3 had an ability to self-govern the pH for solventogenic fermentation, thus the absence of external pH control did not affect its performance. However, the pH control is compulsory for cellulose and xylan setups due to the absence of free monosaccharides during the direct conversion of polyssacharides.
7. Most of the solventogenic *Clostridia* do not have a distinct cellulolytic and xylanolytic performance. This study has revealed that a wild-type culture BOH3 has unique properties, e.g., its ability to produce comparable amount of hydrogen in mesophilic condition from cellulose and xylan, in comparison with other thermophilic hydrogen-producing bacteria.
8. The unutilized cellulose and xylan were detected in the end of fermentation. This was due to the absence of pH control during the fermentation course. With the provision of online pH controller, production of hydrogen from cellulose and xylan can be further improved. This led to the lower amount of unutilized

cellulose and xylan in the end of fermentation, thus increased the hydrogen production yield.

9. There were limited studies about the xylanolytic performance from a solventogenic *Clostridia*. This could be due to that the hydrolysate of xylan, xylose, is less preferable for the microorganisms compared with glucose as the hydrolysate of cellulose. Furthermore, xylan was considered as a hindrance for biofuels production, hence it was commonly removed during the delignification process. The findings from this study on the utilization of xylan reveal a paramount to achieve higher biofuel yield from lignocellulosic biomass, since relatively moderate proportion of xylan (~20-25% by dry weight basis) contained in the lignocellulosic biomass.
10. Direct conversion from lignocellulosic biomass is the future avenue for an efficient butanol production. As the cutting-edge process through the single reactor of delignification, enzymatic hydrolysis, and fermentation processes, culture BOH3 exhibited its advantage by directly converting food waste to butanol.
11. Every fermentation process has a specific type of compatible substrates. Hitherto, direct conversion is concluded to be efficient only for starchy materials. A consideration for the substrate competition for fuel and food has put starchy waste materials as the proper substrates, especially the food waste.
12. Previous studies relied on separate delignification and/or exogenous enzymatic addition as hydrolysis processes prior to the fermentation of starchy biomass, such as corn steep liquor, cassava, and sago starch. Besides increasing the production cost of butanol by employing such process chain, the abovementioned substrates are also considered as food substrates. Hence, it will generate the competition of

substrates for biofuels or food. Food waste is not part of the food supply chain and culture BOH3 showed good performance to directly convert it to butanol.

13. High concentration of unutilized substrate was observed in the end of direct conversion process of food waste to butanol. This was due to the inability of culture BOH3 to hydrolyze completely the fermentable substrate in the food waste. Thus, high concentration of substrate was needed to achieve high concentrations of butanol. An investigation to measure the remnant of volatile solid (VS) of the food waste treatment residue must be performed. This is aimed to assess the feasibility for the further process (stabilization process) of food waste residue, such as aerobic composting or anaerobic digestion. Furthermore, various added-value products from the food waste residue can be further exploited, such as compost, methane, hydrogen, etc.
14. Culture BOH3's ability to utilize food waste as the substrate to grow and produce butanol in the absence of YEP, to self-govern pH during the fermentation course in the absence of pH controller has surfaced an avenue for the possible economically affordable butanol production from food waste via biological process. Food waste as an abundant substrate would significantly decrease the substate provision cost (~56% from overall butanol production cost). Furthermore, omission of the costly YEP and absence of pH controller would further decrease the butanol production cost. Thus, these findings promise a further competitive butanol production cost with that produced from petrochemical route in the near future.
15. The sequence of this study was initiated by isolation of culture BOH3, followed by the assessment of its cellulolytic, xylanolytic, and solventogenic properties. Distinct abovementioned properties were further enhanced by high-cell density

inoculation, stepwise dosing of substrate, and yeast extract-peptone (YEP) addition, thus achieved the remarkable butanol production. An industrial application study for culture BOH3 was performed by testing an innovative biofuels production process-direct conversion- with food waste as a model of real waste. Culture BOH3 showed good performance to convert food waste to butanol, despite high concentration of food waste was required. Throughout this sequence of studies, the investigation on the laboratory-scale about the possibility of this culture to be further employed in a pilot- or industrial-scale is relatively fully-elaborated.

6.2. Recommendations

1. Continuous reactor running by performing stepwise dosing of substrate is also recommended. Wild-type culture BOH3 showed its inability to utilize the bulkdosed substrate due to the substrate inhibition. Furthermore, the gradual substrate feeding as in stepwise dosing mode was beneficial due to the more stable transition between acidogenic and solventogenic stages. This would subdue the necessity for the installation of pH controller and neutralizer chemical addition in the solventogenic fermentation, as pH control was governed solely by culture BOH3. A more gentle transition between acidogenic and solventogenic stages occur due to the adequate time for the culture to re-assimilate the acids to solvents, since the gradual and slower rate of pH decrease would be experienced by the culture.
2. Addition of the costly YEP subdued the competitiveness of butanol production via biological route. This study showed an omission of YEP would be amenable with the expense of stepwise dosing of substrate and augmented-inoculation method

application. As this is the first report for this phenomenon in batch bottle, a further investigation through reactor running experiment is required to confirm this finding.

3. The ability of culture BOH3 in attaching as biofilm to the supportive solid surface needs to be investigated. An attached biological growth in solid surface, such as polyurethane, would increase the cell density and minimize the cell wash out from the reactor.
4. A combination of high cell density application and stepwise dosing of substrate, equipped with attached biological growth, would govern a robust fermentation system. Thus, the overall fermentation process can be improved and production cost for butanol can be further suppressed.
5. Hydrogen production as the main product from the direct conversion of cellulose and xylan can be further improved by performing pH maintenance in the fermentation system. pH maintenance will favor a better environment for culture BOH3 in utilizing cellulose and xylan as the substrates, thus the culture can utilize higher proportion of the substrates and the final concentration of produced hydrogen can be improved. pH maintenance at neutral range would be an optimum working range for the cellulase and xylanase to perform the hydrolysis process.
6. Incremental of cell density will improve the performance of the direct conversion process of cellulose and xylan, concurrently with the provision of on line pH control system. This will fasten the hydrolysis process, in which hydrogen production is coupled with volatile fatty acids production. Employing augmented-inoculation setup without an online pH control system would offset the hydrolysis

process, due to rapid acids production and pH decrease in the fermentation system.

7. Culture BOH3 showed good performance for the direct conversion of food waste to butanol. However, high amount of food waste was required to achieve high concentration of butanol that impacted to the high amount of unutilized substrate at the end of fermentation. The residue from the fermentation that still contained high volatile solid (VS) can be further converted to other value-added products, such as compost, methane, hydrogen, etc.. In order to improve the fermentation efficiency, a better separation of the food waste should be performed.
8. During this study, only inorganic waste and chunky-size waste that were separated from the food waste, such as rubber band, plastic, shrimp shell, chicken bone, etc. The latter mentioned contents were commonly found in the typical food waste composition, however, culture BOH3 was unable to ferment those compounds. Finer waste sorting is required to allow only starchy waste materials to be included as the substrates from food waste, such as rice, corn, potato, noodle, etc. Thus, it is expected to achieve a higher butanol yield or comparable amount of butanol with lesser concentration of food waste as the substrate.

   Autoclaving of the food waste prior to the fermentation experiment was conducted. This method provided a sterilized food waste that conditioned a pure culture fermentation system. However, provision of high energy due to the autoclaving would increase the energy requirement. This can be overcome by spiking higher concentration of sodium acetate (60-90 mM) and decrease the pH (~4.5). The abovementioned adjustments would further select the indigenous microorganisms, while culture BOH3 can grow at that specific condition.

Food waste is considered as the most appropriate substrate for direct conversion process to produce butanol, due to its abundance, relatively costless, and not interfering the food supply chain. A bulkwise dosing of food waste is considered to be more appropriate rather than stepwise dosing method, due to the high viscosity of food waste. Based on the experimental results, pH can be set at 5.0-5.5 at the beginning of the process, with the absence of pH controller. Addition of pricey YEP is not considered to be crucial, due to the presence of trace nutrients in the food waste. An attached biological growth of culture BOH3 should be implemented as it can further enhance the fermentation process, due to the lesser amount of cell wash out. The produced hydrogen gas is feasible to be captured due to high quantity and its high heating value, while the produced acetone, butanol, and ethanol can be further separated via a less energy-intensive process, such as in line pervaporative membrane separation. A lower investment cost can be achieved by implementing this overall configuration, due to the absence of pH controller and lower footprint of the reactor due to the faster fermentation rate as an augmented-inoculation method is implemented. Thus, a significant cost reduction can be achieved in order to lower the butanol production cost from the biological process.

REFERENCE

Ahn, J-H., Sang, B-I., Um, Y. 2011. Butanol production from thin stillage using *Clostridium pasteurianum.* Bioresour. Technol., 102(7), 4934–4937.

Ahring, B.K., Jensen, K., Nielsen, P., Bjerre, A.B., Schmidt, A.S. 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. Bioresour. Technol., 58(2), 107-113.

American Public Health Association. 2005. Standard methods for the examination of water and wastewater analysis. 21st edition, Washington D.C.: U.S.A.

Angenent, L.T. 20007. Energy biotechnology: beyond the general lignocellulose-to-ethanol pathway. Curr. Opin. Biotechnol., 18, 191-192.

Asif, M., Muneer, T. 2007. Energy supply, its demand and security issues for developed and emerging economies. Renew. Sust. Energ. Rev., 11(7), 1388-1413.

Assobhei, O., El Kanouni, A., Ismaili, M., Loutfi, M., Petitdemange, H. 1998. Effect of acetic and butyric acids on the stability of solvent and spore formation by *Clostridium acetobutylicum* ATCC824 during repeated subculturing. J. Ferment. Bioeng., 85(2), 209-212.

Atiyeh, H.K., Maddipati, P., Bellmer, D.D., Huhnke, R.L. 2011. Ethanol production from syngas by *Clostridium* strain P11 using corn steep liquor as a nutrient replacement to yeast extract. Bioresour. Technol. 102(11), 6494-6501.

Balat, M., Balat, H. 2009. Recent trends in global production and utilization of bio-ethanol fuel. Appl. Energ, 86, 2273-2282.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J. 2006. Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J. Biotechnol., 125(2), 198-209.

Bobleter, O. 1994. Hydrothermal degradation of polymers derived from plants. Prog. Polym. Sci., 19(5), 797-841.

Boemi, S., Papadopoulos, A., Karagiannidis, A., Kontogianni, S. 2010. Barriers on the propagation of renewable energy sources and sustainable solid waste management practices in greece. Waste Manag. Res., 28(11), 967-976.

Bon, R. 2005. The end of cheap oil. Technol Rev, 108(11), 1-10.

Bond, K., Stutzenberger, F. 1989. A note on the localization of cellulosome formation in *Thermomonospora curvata*. J. Appl. Bacteriol., 67(6), 605-609.

Borden, J.R., Papoutsakis, E.T. 2007. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Appl. Environ. Microbiol., 73(9), 3061-3068.

Bowles, L.K., Ellefson, W.L. 1985. Effects of butanol on *Clostridium acetobutylicum*. Appl. Environ. Microbiol., 50(5), 1165-1170.

Bresticgoachet, N., Gunasekaran, P., Cami, B., Baratti, J.C. 1989. Transfer and expression of an *Erwinia chrysanthemi* cellulase gene in *Zymomonas mobilis*. J. Gen. Microbiol., 135, 893-902.

Brown, R.M., Saxena, I.M. 2000. Cellulose biosynthesis: A model for understanding the assembly of biopolymers. Plant Physiol. Bioch., 38(1-2), 57-67.

Bryant, D.L., Blaschek, H.P. 1988. Buffering as a means for increasing growth and butanol production by *Clostridium acetobutylicum*. J Ind Microbiol, 3(1), 49-55.

Campos, E.J., Qureshi, N., Blaschek, H.P. 2002. Production of acetone butanol ethanol from degermed corn using *Clostridium beijerinckii* BA101. Appl. Biochem. Biotech. 98-100(1-9), 553-561.

Carlile, M.J., and S. C. Watkinson. 1997. *The fungi*. Academic Press, New York, U.S.A.

Chang, H.N., Yoo, I.K., Kim, B.S. 1994. High-density cell-culture by membrane-based cell recycle. Biotechnol. Adv., 12(3), 467-487.

Chang, V.S., Holtzapple, M.T. 2000. Fundamental factors affecting biomass enzymatic reactivity. Appl. Biochem. Biotechnol., 84-86, 5-37.

Cheirsilp, B., Tran, H.T.M., Hodgson, B., Umsakul, K. 2010. Potential use of *Bacillus subtilis* in a co-culture with *Clostridium butylicum* for acetone-butanol-ethanol production from cassava starch. Biochem. Eng. J., 48(2), 260-267.

Chiao, J.S., Sun, Z.H. 2007. History of the acetone-butanol-ethanol fermentation industry in china: Development of continuous production technology. J. Mol. Microbiol. Biotechnol., 13(1-3), 12-14.

Cherubini, F., Bird, N.D., Cowie, A., Jungmeier, G., Schlamadinger, B., Gallasch, SW. 2009. Energy- and greenhouse gas-based LCA of biofuel and bioenergy systems: Key issues, ranges and recommendations. Resour. Recov. Conserv., 53, 434–447.

Chow, J., Kopp, R.J., Portney, P.R. 2003. Energy resources and global development. Science, 302(5650), 1528-1531.

Chum, H.L., Johnson, D.K., Black, S., Baker, J., Grohmann, K., Sarkanen, K.V., Wallace, K., Schroeder, H.A. 1988. Organosolv pretreatment for enzymatic

hydrolysis of poplars: I. Enzyme hydrolysis of cellulosic residues. Biotechnol. Bioeng., 31(7), 643-649.

Claassen, P.A.M., van Lier, J.B., Contreras, A.M.L., van Niel, E.W.J., Sijtsma, L., Stams, A.J.M., de Vries, S.S., Weusthuis, R.A. 1999. Utilisation of biomass for the supply of energy carriers. Appl. Microbiol. Biot., 52(6), 741-755.

Cock, J.H. 1982. Cassava - a basic energy-source in the tropics. Science, 218(4574), 755-762.

Cowling, E.B., Kirk, T.K. 1976. Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion processes. Biotechnol. Bioeng., (6), 95-123.

Daniels, L., Sparling, R., Sprott, G.D. 1984. The bioenergetics of methanogenesis. Biochim Biophys. Acta, 768(2), 113-163.

Das, D., Veziroglu, T.N. 2001. Hydrogen production by biological processes: A survey of literature. Int. J. Hydrogen Energ., 26(1), 13-28.

Datta, R., Ranganathan, V.T. 2002. Variable-speed wind power generation using doubly fed wound rotor induction machine-a comparison with alternative schemes. Energ. Convers., 17(3), 414-421.

Demattos, M.J.T., Deboer, J.P., Zoutberg, G.R., Neijssel, O.M. 1994. Metabolic shift analysis at high cell densities. FEMS Microbiol. Rev., 14(1), 21-28.

Demirbas, A. 2001. Biomass resource facilities and biomass conversion processing for fuels and chemicals. Energ. Convers. Manage., 42(11), 1357-1378.

Desai, R.P., Papoutsakis, E.T. 1999. Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum.* Appl. Environ. Microb., 65(3), 936-945.

de Vries, B.J.M, van Vuuren, D.P., Hoogwijk, M.M. 2007. Renewable energy sources: Their global potential for the first-half of the 21st century at a global level: An integrated approach. Energ. Policy, 35, 2590–2610.

Demirbas, M.F., Balat, M., Balat, H. 2009. Potential contribution of biomass to the sustainable energy development. Energ. Convers. Manage., 50, 1746–1760.

Dincer, I. 2000. Renewable energy and sustainable development: a crucial review. Renew. Sust. Energ. Rev. 4(2), 157-175.

Doi, R.H., Park, J.S., Liu, C.C., Malburg, L.M., Tamaru, Y., Ichiishi, A., Ibrahim, A. 1998. Cellulosome and noncellulosomal cellulases of *Clostridium cellulovorans*. Extremophiles, 2(2), 53-60.

Duff, S.J.B., Murray, W.D. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. Bioresour. Technol., 55(1), 1-33.

Dumon, C., Song, L., Bozonnet, S., Fauré, R., O'Donohue, M.J. 2011. Progress and future prospects for pentose-specific biocatalysts in biorefining. Process Biochem., *In press.*

Dürre, P. 2005. *Handbook on Clostridia*. CRC Press, Boca Raton.

Dürre, P. 2007. Biobutanol: An attractive biofuel. Biotechnol. J., 2(12), 1525-1534.

Dürre, P. 2008. Fermentative butanol production - bulk chemical and biofuel. Ann. N. Y. Acad. Sci., 1125, 353-362.

Dürre, P. 1998. New insights and novel developments in Clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biot., 49(6), 639-648.

Espey, S. 2001. Renewables portfolio standard: a means for trade with electricity from renewable energy sources? Energ. Policy, 29, 557-566.

Esterbauer, H., Steiner, W., Labudova, I., Hermann, A., Hayn, M. 1991. Production of *Trichoderma* cellulase in laboratory and pilot scale. Bioresour. Technol., 36(1), 51-65.

Gabriel, C.L. 1928. Butanol fermentation process. Ind. Eng. Chem., 28: 1063–1067.

Excoffier, G., Toussaint, B., Vignon, M.R. 1991. Saccharification of steam-exploded poplar wood. Biotechnol. Bioeng., 38(11), 1308-1317.

Ezeji, T., Blaschek, H.P. 2008. Fermentation of dried distillers' grains and solubles (DDGS) hydrolysates to solvents and value-added products by solventogenic *Clostridia*. Bioresour. Technol., 99(12), 5232-5342.

Ezeji, T., Qureshi, N., Blaschek, H.P. 2007a. Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. Biotechnol. Bioeng., 97(6), 1460-1469.

Ezeji, T.C., Qureshi, N., Blaschek, H.P. 2004a. Acetone butanol ethanol (ABE) production from concentrated substrate: Reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. Appl. Microbiol. Biotechnol., 63(6), 653-658.

Ezeji, T.C., Qureshi, N., Blaschek, H.P. 2007b. Bioproduction of butanol from biomass: From genes to bioreactors. Curr. Opin. Biotechnol., 18(3), 220-227.

Ezeji, T.C., Qureshi, N., Blaschek, H.P. 2004b. Butanol fermentation research: Upstream and downstream manipulations. Chem. Rec., 4(5), 305-314.

Fan, L.T., Gharpuray, M.M., Lee, Y.H. 1987. *Cellulose hydrolysis*. Springer-Verlag, Berlin, Germany.

Fan, L.T., Lee, Y.H., Beardmore, D.H. 1980. Mechanism of the enzymatic-hydrolysis of cellulose - effects of major structural features of cellulose on enzymatic-hydrolysis. Biotechnol. Bioeng., 22(1), 177-199.

Farhat, A., Normand, L., Chavez, E.R., Touchburn, S.P. 1998. Nutrient digestibility in food waste ingredients for Pekin and Muscovy ducks. Poultry Sci., 77(9), 1371-1376

Fengel, D., Wegener, G. 1984. *Wood: Chemistry, ultrastructure, reactions*. De Gruyter, Berlin.

Fields, M.W., Russell, J.B., Wilson, D.B. 1998. The role of ruminal carboxymethylcellulases in the degradation of beta-glucans from cereal grain. FEMS Microbiol. Ecol., 27(3), 261-268.

Fond, O., Engasser, J.M., Matta-El-Amouri, G., Petitdemange, H. 1986. The acetone butanol fermentation on glucose and xylose. I. Regulation and kinetics in batch cultures. Biotechnol. Bioeng., 28(2), 160-166.

Fond, O., Mattaammouri, G., Petitdemange, H., Engasser, J.M. 1985. The role of acids on the production of acetone and butanol by *Clostridium acetobutylicum.* Appl. Microbiol. Biot. 22(3), 195-200.

Fouad, M., Abouzeid, A.A., Yassein, M. 1976. Fermentative production of acetone-butanol by *Clostridium acetobutylicum.* Acta Biol. Acad. Sci. H., 27(2-3), 107-117.

Gal, L., Pages, S., Gaudin, C., Belaich, A., Reverbel-Leroy, C., Tardif, C., Belaich, J.P. 1997. Characterization of the cellulolytic complex (cellulosome) produced by *Clostridium cellulolyticum.* Appl. Environ. Microbiol., 63(3), 903-909.

Garrote, G., Dominguez, H., Parajo, J.C. 1999. Hydrothermal processing of lignocellulosic materials. Holz Roh Werkst, 57(3), 191-202.

Gomez, L.D., Steele-King, C.G., McQueen-Mason, S.J. 2008. Sustainable liquid biofuels from biomass: The writing's on the walls. New Phytol., 178(3), 473-485.

Goyal, H.B., Seal, D., Saxena, R.C. 2008. Bio-fuels from thermochemical conversion of renewable resources: A review. Renew. Sust. Energ. Rev., 12(2), 504-517.

Grabber, J.H. 2005. How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci., 45(3), 820-831.

Gregg, D., Saddler, J.N. 1996a. A techno-economic assessment of the pretreatment and fractionation steps of a biomass-to-ethanol process. Appl. Biochem. Biotech., 57-8, 711-727.

Gregg, D.J., Saddler, J.N. 1996b. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. Biotechnol. Bioeng., 51(4), 375-383.

Grethlein, H.E. 1985. The effect of pore-size distribution on the rate of enzymatic-hydrolysis of cellulosic substrates. Bio-Technol, 3(2), 155-160.

Grobben, N.G., Eggink, G., Cuperus, F.P., Huizing, H.J. 1993. Production of acetone, butanol and ethanol (ABE) from potato wastes - fermentation with integrated membrane extraction. Appl. Microbiol. Biot., 39(4-5), 494-498.

Groot, W.J., van der Lans, R.G.J.M., Luyben, K.Ch.A.M. 1992. Technologies for butanol recovery integrated with fermentations. Process Biochem., 27(2), 61-75.

Gu, Y., Hu, S.Y., Chen, J., Shao, L.J., He, H.Q., Yang, Y.L., Yang, S., Jiang, W.H. 2009. Ammonium acetate enhances solvent production by *Clostridium acetobutylicum* EA2018 using cassava as a fermentation medium. J. Ind. Microbiol. Biot., 36(9), 1225-1232.

Gu, Y, Jiang, Y. Wu, H., Liu, X., Li, Z., Li, J., Xiao, H., Shen, Z., Dong, H., Yang, Y., Li, Y., Jiang, W., Yang, S. 2011. Economical challenges to microbial producers of butanol: feedstock, butanol ratio and titer. Biotech. J., 6, 1348-1357.

Guerlava, P., Izac, V., Tholozan, J.L. 1998. Comparison of different methods of cell lysis and protein measurements in *Clostridium perfringens*: Application to the cell volume determination. Curr. Microbiol., 36(3), 131-135.

Gutierrez, N.A., Maddox, I.S., Schuster, K.C., Swoboda, H., Gapes, J.R. 1998. Strain comparison and medium preparation for the acetone-butanol-ethanol (ABE) fermentation process using a substrate of potato. Bioresour. Technol., 66(3), 263-265.

Harris, L.M., Desai, R.P., Welker, N.E., Papoutsakis, E.T. 2000. Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? Biotechnol. Bioeng., 67(1), 1-11.

He, J., Ritalahti, K.M., Yang, K.L., Koenigsberg, S.S., Loffler, F.E. 2003. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature, 424(6944), 62-65.

Heap, J.T., Pennington, O.J., Cartman, S.T., Carter, G.P., Minton, N.P. 2007. The clostron: A universal gene knock-out system for the genus *Clostridium*. J. Microbiol. Methods, 70(3), 452-464.

Hendriks, A.T.W.M., Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol., 100(1), 10-18.

Higashide, W., Li, Y., Yang, Y., Liao, J.C. 2011. Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. Appl. Environ. Microbiol., 77(8), 2727-2733.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels. Science, 315, 804-807.

Hoogwijk, M., Faaij, A., van den Broek, R., Berndes, G., Gielen, D., Turkenburg, W. 2003. Exploration of the ranges of the global potential of biomass for energy. Biomass Bioenerg., 25(2), 119-133.

Höök, M., Hirsch, R., Aleklett, K. 2009. Giant oil field decline rates and theirinfluence on world oil production. Energ. Policy, 37(6), 2262-2272.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, Inc., New York, U.S.A.

Janati-Idrissi, R., Junelles, A-M., Kanouni, A.E., Petitdemange, H. Gay, R. 1989. Pyruvate fermentation by *Clostridium acetobutylicum*. Biochem. Cell. Biol., 67(10), 735-739.

Jesse, T.W., Ezeji, T.C., Qureshi, N., Blaschek, H.P. 2002. Production of butanol from starch-based waste packing peanuts and agricultural waste. J. Ind. Microbiol. Biot., 29(3), 117-123.

Johansson, D.J.A., Azar, C. 2007. A scenario based analysis of land competition between food and bioenergy production in the us. Climatic Change, 82(3-4), 267-291.

Jones, D.T. 2001. Applied acetone-butanol fermentation. *In Clostridia*. Biotechnological and medical applications. Bahl, H., Dürre, P., Eds.: 125–168.Weinheim, Germany: Wiley-VCH Jones, D.T., Keis, S. 1995. Origins and relationships of industrial solvent-producing Clostridial strains. FEMS Microbiol. Rev., 17(3), 223-232.

Jones, D.T., Shirley, M., Wu, X.Y., Keis, S. 2000. Bacteriophage infections in the industrial acetone butanol (AB) fermentation process. J. Mol. Microb. Biotech., 2(1), 21-26.

Jones, D.T., Woods, D.R. 1986. Acetone-butanol fermentation revisited. Microbiol. Rev., 50(4), 484-524.

Kabyemela, B.M. Takigawa, M., Adschiri, T., Malaluan, R.M., Arai, K. 1998. Mechanism and kinetics of cellobiose decomposition in sub- and supercritical water. Ind. Eng. Chem. Res., 37 (2), 357–361.

Kataeva, I.A., Yang, S.J., Dam, P., Poole, F.L., 2nd, Yin, Y., Zhou, F., Chou, W.C., Xu, Y., Goodwin, L., Sims, D.R., Detter, J.C., Hauser, L.J., Westpheling, J., Adams, M.W. 2009. Genome sequence of the anaerobic, thermophilic, and

cellulolytic bacterium *Anaerocellum thermophilum* DSM 6725. J. Bacteriol., 191(11), 3760-3761.

Kesicki, F. 2010. The third oil price surge - what's different this time? Energ Policy, 38(3), 1596-1606.

Khan, A. W., Meek, E., Sowden, L.C., Colvin, J.R. 1994. Emendation of genus *Acetivibrio* and description of *Acetivibrio cellulosolvens*, new species, of nonmotile cellulolytic mesophile. Int. J. Syst. Bacteriol., 34, 410–422.

Khanal, S.K., Chen, W.H., Li, L., Sung, S.W. 2004. Biological hydrogen production: Effects of pH and intermediate products. Int. J. Hydrogen Energy, 29(11), 1123-1131.

Khoo, H.H., Lim, T.Z., Tan, R.B.H. 2010. Food waste conversion options in Singapore: Environmental impacts based on an LCA perspective. Sci. Total Environ., 408(6), 1367-1373.

Klass, D.L. 1998. Biomass for Renewable Energy, Fuels, and Chemicals. Academic Press: New York, U.S.A.

Kim, B.H., Bellows, P., Datta, R., Zeikus, J.G. 1984. Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: Utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. Appl. Environ. Microbiol., 48(4), 764-770.

Koopmans, A. 2005. Biomass energy demand and supply for South and South-East Asia—assessing the resource base. Biomass Bioenerg., 28(2), 133-150.

Kraemer, J.T., Bagley, D.M. 2007. Improving the yield from fermentative hydrogen production. Biotechnol. Lett., 29(5), 685-695.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res., 48, 3713–3729

Kumar, M., Gayen, K. 2011. Developments in biobutanol production: New insights. Appl. Energ, 88(6), 1999-2012.

la Grange, D.C., den Haan, R., van Zyl, W.H. 2010. Engineering cellulolytic ability into bioprocessing organisms. Appl. Microbiol. Biot., 87(4), 1195-1208.

Laureano-Perez, L., Teymouri, F., Alizadeh, H., Dale, B.E. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. Appl. Biochem. Biotech., 121, 1081-1099.

Lee, J., 1997. Biological conversion of lignocellulosic biomass to ethanol. J. Biotechnol., 56(1), 1-24.

Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., Jung, K.S. 2008. Fermentative butanol production by *Clostridia*. Biotechnol. Bioeng., 101(2), 209-228.

Levin, B.R., Stewart, F.M. 1977. Probability of establishing chimeric plasmids in natural-populations of bacteria. Science, 196(4286), 218-220.

Lewin, R. 1982. Can genes jump between eukaryotic species. Science, 217(4554), 42-43.

Lin, C. Urbance, J.W., Stahl, D.A. 1994. *Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens* are members of the greater Clostridial assemblage. FEMS Microbiol. Lett., 124, 151–155.

Lipnizki, F., Hausmanns, S., Laufenberg, G., Field, R., Kunz, B. 2000. Use of pervaporation-bioreactor hybrid processes in biotechnology. Chem. Eng. Technol., 23(7), 569-577.

Liu, S.J., Amidon, T.E., Wood, C.D., Shupe, A.M., Wang, Y., Graves, M. 2008. Biorefinery: Conversion of woody biomass to chemicals, energy and materials. J. Biobased Mater. Bio., 2(2), 100-120.

Liu, C.G., Wyman, C.E. 2003. The effect of flow rate of compressed hot water on xylan, lignin, and total mass removal from corn stover. Ind. Eng. Chem. Res., 42(21), 5409-5416.

Liu, S. 2010. Woody biomass: Niche position as a source of sustainable renewable chemicals and energy and kinetics of hot-water extraction/hydrolysis. Biotechnol. Adv., 28(5), 563-582.

Liu, Y., Yu, P., Song, X., Qu, Y.B. 2008. Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. Int. J. Hydrogen Energy, 33(12), 2927-2933.

Lo, Y.C., Bai, M.D., Chen, W.M., Chang, J.S. 2008. Cellulosic hydrogen production with a sequencing bacterial hydrolysis and dark fermentation strategy. Bioresour. Technol., 99(17), 8299-8303.

Lobell, D.B., Burke, M.B., Tebaldi, C., Mastrandrea, M.D., Falcon, W.P., Naylor, R.L. 2008. Prioritizing climate change adaptation needs for food security in 2030. Science, 319(5863), 607-610.

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E. 2008. How biotech can transform biofuels. Nature Biotechnol., 26(2), 169-172.

Lynd, L.R., van Zyl, W.H., McBride, J.E., Laser, M. 2005. Consolidated bioprocessing of cellulosic biomass: An update. Curr Opin Biotech, 16(5), 577-583.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol R, 66(3), 506-+.

Madihah, M.S., Ariff, A.B., Sahaid, K.M., Suraini, A.A., Karim, M.I.A. 2001. Direct fermentation of gelatinized sago starch to acetone-butanol-ethanol by *Clostridium acetobutylicum.* World J. Microb. Biot., 17(6), 567-576.

Magnani, F., Mencuccini, M., Borghetti, M., Berbigier, P., Berninger, F., Delzon, S., Grelle, A., Hari, P., Jarvis, P.G., Kolari, P., Kowalski, A.S., Lankreijer, H., Law, B.E., Lindroth, A., Loustau, D., Manca, G., Moncrieff, J.B., Rayment, M., Tedeschi, V., Valentini, R., Grace, J. 2007. The human footprint in the carbon cycle of temperate and boreal forests. Nature, 447(7146), 848-850.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., U.S.A.

McMillan, J.D. 1994. Pretreatment of lignocellulosic biomass. In *Enzymatic Conversion of Biomass for Fuels Production;* Himmel, M.E., Baker, J.O., Overend, R.P., Eds.; ACS: Washington DC, U.S.A., 292-324.

Mermelstein, L.D., Papoutsakis, E.T., Petersen, D.J., Bennett, G.N. 1993. Metabolic engineering of *Clostridium acetobutylicum* ATCC824 for increased solvent production by enhancement of acetone formation enzyme-activities using a synthetic acetone operon. Biotechnol. Bioeng., 42(9), 1053-1060.

Mielenz, R. 2001. Ethanol production from biomass: technology and commercialization status. Curr. Opin. Microbiol., 4(3), 324-329.

Mladenovska, Z., Mathrani, I.M., Ahring, B.K. 1995. Isolation and characterization of *Caldicellulosiruptor lactoaceticus* sp-nov, an extremely thermophilic, cellulolytic, anaerobic bacterium. Arch Microbiol, 163(3), 223-230.

Moniruzzaman, M., Dien, B.S., Skory, C.D., Chen, Z.D., Hespell, R.B., Ho, N.W.Y., Dale, B.E., Bothast, R.J. 1997. Fermentation of corn fibre sugars by an engineered xylose utilizing *Saccharomyces* yeast strain. World J. Microb. Biot., 13(3), 341-346.

Montoya, D., Arevalo, C., Gonzales, S., Aristizabal, F., Schwarz, W.H. 2001. New solvent-producing *Clostridium* sp. Strains, hydrolyzing a wide range of polysaccharides, are closely related to *Clostridium butyricum.* J. Ind. Microbiol. Biotechnol., 27(5), 329-335.

Montoya, D., Spitia, S., Silva, E., Schwarz, W.H. 2000. Isolation of mesophilic solvent-producing *Clostridia* from Colombian sources: physiological characterization, solvent production and polysaccharide hydrolysis. J. Biotechnol., 79, 117-126.

Moreira, A.R., Ulmer, D.C., Linden, J.C. 1981. Butanol toxicity in the butylic fermentation. Biotechnol. Bioeng., 567-579.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol., 96(6), 673-686.

Myhr, A.I., Traavik, T. 1999. The Precautionary Principle Applied to Deliberate Release of Genetically Modified Organisms (GMOs). Microb. Ecol. Health D., 11(2), 65-74.

Nair, R.V., Bennett, G.N., Papoutsakis, E.T. 1994. Molecular characterization of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC824. J. Bacteriol., 176(3), 871-885.

Ng, H.Y., Hermanowicz, S.W. 2005. Membrane bioreactor operation at short solids retention times: Performance and biomass characteristics. Water Res., 39(6), 981-992.

Ng, T.K., Ben-Bassat, A., Zeikus, J.G. 1981. Ethanol production by thermophilic bacteria: Fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. Appl. Environ. Microbiol., 41(6), 1337-1343.

Nguyen, T.A.D., Kim, J.P., Kim, M.S., Oh, Y.K., Sim, S.J. 2008. Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritima* and *Thermotoga neapolitana* in batch fermentation. Int. J. Hydrogen Energy, 33(5), 1483-1488.

Ni, Y., Sun, Z.H. 2009. Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. Appl. Microbiol. Biot., 83(3), 415-423.

Nuvolari, A. 2004. Collective invention during the British industrial revolution: the case of the Cornish pumping engine. Camb. J. Econ., 28(3), 347-363.

Rainey, F.A., Donnison, A.M., Janssen, P.H., Saul, D., Rodrigo, A., Bergquist, P.L., Daniel, R.M., Stackebrandt, E., Morgan, H.W. 1994. Description of *Caldicellulosiruptor saccharolyticus* gen. nov., sp. nov: an obligately anaerobic, extremely thermophilic, cellulolytic bacterium. FEMS Microbiol. Lett., 120, 263–266.

Palonen, H., Thomsen, A.B., Tenkanen, M., Schmidt, A.S., Viikari, U. 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. Appl. Biochem. Biotech. 117(1), 1-17.

Parekh, M., Formanek, J., Blaschek, H.P. 1999. Pilot-scale production of butanol by *Clostridium beijerinckii* BA101 using a low-cost fermentation medium based on corn steep water. Appl. Microbiol. Biot., 51(2), 152-157.

Pehnt, M. 2006. Dynamic life cycle assessment (LCA) of renewable energy technologies. Renew. Energy., 31(1), 55-71.

Pimentel, D., McNair, M., Duck, L., Pimentel, M., Kamil, J. 1997. The value of forests to world food security. Hum. Ecol., 25(1), 91-120.

Pimentel, D., Michele, H., Glickstein, Zimmerman, M.,, Allen, R., Becker, K., Evans, J., Hussain, B., Sarsfeld, R., Grosfeld, A., Seidel, T. 2002. Renewable Energy: Current and Potential Issues. BioScience, 52(12): 1111-1120.

Pitkanen, J.P., Rintala, E., Aristidou, A., Ruohonen, L., Penttila, M. 2005. Xylose chemostat isolates of *Saccharomyces cerevisiae* show altered metabolite and enzyme levels compared with xylose, glucose, and ethanol metabolism of the original strain. Appl. Microbiol.Biotechnol., 67(6), 827-837.

Qureshi, N., Blaschek, H.P. 2000a. Butanol production using *Clostridium beijerinckii* BA101 hyper-butanol producing mutant strain and recovery by pervaporation. Appl. Biochem. Biotech., 84-6, 225-235.

Qureshi, N., Blaschek, H.P. 2000b. Economics of butanol fermentation using hyper-butanol producing *Clostridium beijerinckii* BA101. Food Bioprod. Process., 78(C3), 139-144.

Qureshi, N., Blaschek, H.P. 2001. Evaluation of recent advances in butanol fermentation, upstream, and downstream processing. Bioproc. Biosyst. Eng., 24(4), 219-226.

Qureshi, N., Blaschek, H.P. 1999. Production of acetone butanol ethanol (ABE) by a hyper-producing mutant strain of *Clostridium beijerinckii* BA101 and recovery by pervaporation. Biotechnol. Prog., 15(4), 594-602.

Qureshi, N., Hughes, S., Maddox, I.S., Cotta, M.A. 2005. Energy-efficient recovery of butanol from model solutions and fermentation broth by adsorption. Bioprocess Biosyst. Eng., 27(4), 215-222.

Qureshi, N., Saha, B.C., Hector, R.E., Hughes, S.R., Cotta, M.A. 2008. Butanol production from wheat straw by simultaneous saccharification and fermentation using *Clostridium beijerinckii*: part I—batch fermentation. Biomass and Bioenerg., 32, 168-175.

Ramos, L.P. 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Quim Nova, 26(6), 863-871.

Ramos, L.P., Breuil, C., Saddler, J.N. 1993. The use of enzyme recycling and the influence of sugar accumulation on cellulose hydrolysis by *Trichoderma*-cellulases. Enzyme Microb. Tech., 15(1), 19-25.

Ranatunga, T.D., Jervis, J., Helm, R.F., McMillan, J.D., Wooley, R.J. 2000. The effect of overliming on the toxicity of dilute acid pretreated lignocellulosics: The role of inorganics, uronic acids and ether-soluble organics. Enzyme Microb. Technol., 27(3-5), 240-247.

Rathmann, R., Szklo, A., Schaeffer, R. 2010. Land use competition for production of food and liquid biofuels: An analysis of the arguments in the current debate. Renew. Energ., 35(1), 14-22.

Reese, E.T., Siu, R.G., Levinson, H.S. 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol., 59(4), 485-497.

Reese, E.T., Smakula, E., Perlin, A.S. 1959, Enzymic production of cellotriose from cellulose. Arch. biochem. biophys., 85., 171-175.

Richards, B.S. 2006. Enhancing the performance of silicon solar cells via the application of passive luminescence conversion layers. Sol. Energ. Mat. Sol. C., 90(15), 2329-2337.

Rose, A.H. 1961. Industrial Microbiology. London, U.K.: Butterworths.

Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V. 2005. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnol. Prog., 21(3), 816-822.

Sanchez, C. R., Peres, C.S., Barbosa, H.R. 1999. Growth and endoglucanase activity of *Acetivibrio cellulolyticus* grown in three different cellulosic substrates. Rev. Microbiol., 30, 310–314.

Sathitsuksanoh, N., Zhu, Z., Ho, T-J., Bai, M-D., Zhang, Y-H.P. 2010. Bamboo saccharification through cellulose solvent-based biomass pretreatment followed by enzymatic hydrolysis at ultra-low cellulase loadings. Bioresour. Technol. 101, 4926-4929.

Sato, K.J., Goto, S.G., Yonemura, S.T., Sekine, K.J., Okuma, E.K., Takagi, Y.S., Honnami, K.Y., Saiki, T. 1992. Effect of yeast extract and vitamin-B12 on ethanol-production from cellulose by *Clostridium thermocellum* i-1-b. Appl. Environ. Microbiol., 58(2), 734-736.

Schwarz, W.H. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl Microbiol Biot, 56(5-6), 634-649.

Schuster, K.C., van den Heuvel, R., Gutierrez, N.A., Maddox, I.S. 1998. Development of markers for product formation and cell cycle in batch cultivation of *Clostridium acetobutylicum* ATCC 824. Appl. Microbiol. Biotechnol., 49(6), 669-676.

Scotcher, M.C., Bennett, G.N. 2005. Spoiie regulates sporulation but does not directly affect solventogenesis in *Clostridium acetobutylicum* ATCC824. J. Bacteriol., 187(6), 1930-1936.

Shaw, A.J., Podkaminer, K.K., Desai. S.G., Bardslev, J.S., Rogers, S.R., Thorne, P.G., Hogsett, D.A., Lynd, L.R. (2008). Metabolic engineering of a

thermophilic bacterium to produce ethanol at high yield. PNAS. 105(37), 13769-13774.

Somrutai, W., Takagi, M., Yoshida, T. 1996. Acetone-butanol fermentation by *Clostridium aurantibutyricum* ATCC17777 from a model medium for palm oil mill effluent. J. Ferment. Bioeng., 81(6), 543-547.

Srivastava, R., Kumar, G.P., Srivastava, K.K. 1995. Construction of a recombinant cellulolytic *Escherichia coli*. Gene, 164(1), 185-186.

Sun, Y., Cheng, J.Y. 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour. Technol., 83(1), 1-11.

Suvorov, M., Kumar, R., Zhang, H., Hutcheson, S. 2011. Novelties of the cellulolytic system of a marine bacterium applicable to cellulosic sugar production. Biofuels, *In press*.

Svetlichnyi, V.A., Svetlichnaya, T.P., Chernykh, N.A., Zavarzin, G.A. 1990. *Anaerocellum thermophilum* gen-nov sp-nov - an extremely thermophilic cellulolytic *Eubacterium* isolated from hot-springs in the valley of geysers. Microbiology+, 59(5), 598-604.

Swana, J., Yang, Y., Behnam, M., Thompson, R. 2011. An analysis of net energy production and feedstock availability for biobutanol and bioethanol. Bioresour. Technol., 102 (2), 2112–2117.

Taherzadeh, M.J., Karimi, K. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int. J. Mol. Sci., 9(9), 1621-1651.

Tashiro, Y., Takeda, K., Kobayashi, G., Sonomoto, K. 2005. High production of acetone–butanol–ethanol with high cell density culture by cell-recycling and bleeding. J. Biotechnol. 120(2), 197-206.

Tassinari, T.H., Macy, C.F., Spano, L.A. 1982. Technology advances for continuous compression milling pretreatment of lignocellulosics for enzymatic hydrolysis. Biotechnol. Bioeng., 24(7), 1495-1505.

Tawfik, A., Salem, A., El-Qelish, M. 2011. Two stage anaerobic baffled reactors for bio-hydrogen production from municipal food waste. Bioresour. Technol.

Teeri, T.T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C., Jones, T.A. 1998. Trichoderma reesei cellobiohydrolases: Why so efficient on crystalline cellulose? Biochem. Soc. T., 26(2), 173-178.

Thang, V.H., Kanda, K., Kobayashi, G. 2010. Production of acetone-butanol-ethanol (ABE) in direct fermentation of cassava by *Clostridium saccharoperbutylacetonicum* N1-4. Appl. Biochem. Biotechnol., 161(1-8), 157-170.

Thassitou, P.K., Arvanitoyannis, I.S., 2001. Bioremediation: a novel approach to food waste management. Trends Food Sci. Tech., 12(5-6), 185-196.

Tilman, D., Socolow, R., Foley, J.A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C., Williams, R. 2009. Energy. Beneficial biofuels--the food, energy, and environment trilemma. Science, 325(5938), 270-271.

Tsai, S.L., Oh, J., Singh, S., Chen, R.Z., Chen, W. 2009. Functional assembly of minicellulosomes on the *Saccharomyces cerevisiae* cell surface for cellulose hydrolysis and ethanol production. Appl. Environ. Microb., 75(19), 6087-6093.

Umikalsom, M.S., Ariff, A.B., Shamsuddin, Z.H., Tong, C.C., Hassan, M.A., Karim, M.I.A. 1997. Production of cellulase by a wild strain of *Chaetomium globosum* using delignified oil palm empty-fruit-bunch fibre as substrate. Appl. Microbiol. Biot., 47(5), 590-595.

Wachinger, G., Bronnenmeier, K., Staudenbauer, W.L., Schrempf, H. 1989. Identification of mycelium-associated cellulase from *Streptomyces reticuli*. Appl. Environ. Microb., 55(10), 2653-2657.

Wang, A., Gao, L., Ren, N., Xu, J., Liu, C. 2009. Bio-hydrogen production from cellulose by sequential co-culture of cellulosic hydrogen bacteria of *Enterococcus gallinarum* G1 and *Ethanoigenens harbinense* B49. Biotechnol. Lett., 31(9), 1321-1326.

Waxman, B.P. 2010. Clinical research, carbon emissions, climate change and Copenhagen - our lifestyle on the line? ANZ J .Surg., 80(10), 678-679.

Wilks, M. 2009. "Greenwash" at the climate change summit in Copenhagen. BMJ, 339, b5616.

Willson, D.B., Irwin, D.C.1999. Genetics and Properties of Cellulases. Adv. Biochem. Eng./Biotechnol., 65, 1-21.

Wolin, E.A., Wolin, M.J., Wolfe, R.S. 1963. Formation of methane by bacterial extracts. J. Biol. Chem., 238, 2882-2886.

Woods, D.R. 1995. The genetic engineering of microbial solvent production. Trends Biotechnol, 13(7), 259-264.

Woodward, A. 2009. Copenhagen, climate change, revolutions and public health. Aust N. Z. J. Public Health, 33(6), 505-506.

Yang, B., Dai, Z., Ding, S-Y., Wyman, C.E. 2011. Enzymatic hydrolysis of cellulosic biomass. Bioefuels. 2(4), 421-450.

Yang, S.J., Kataeva, I., Hamilton-Brehm, S.D., Engle, N.L., Tschaplinski, T.J., Doeppke, C., Davis, M., Westpheling, J., Adams, M.W. 2009b. Efficient

degradation of lignocellulosic plant biomass, without pretreatment, by the thermophilic anaerobe *Anaerocellum thermophilum* DSM 6725. Appl. Environ. Microbiol., 75(14), 4762-4769.

Yang, H., Zhou, Y., Liu, J. 2009. Land and water requirements of biofuel and implications for food supply and the environment in China. Energ. Policy. 37(5), 1876-1885.

Yoo, J.S., Jung, Y.J., Chung, S.Y., Lee, Y.C., Choi, Y.L. 2004. Molecular cloning and characterization of cmcase gene (celc) from *Salmonella typhimurium* ur. J. Microbiol., 42(3), 205-210.

Zhang, Y., Lynd, L.R. 2003. Quantification of cell and cellulase mass concentrations during anaerobic cellulose fermentation: development of an enzyme-linked immunosorbent assay-based method with application to *Clostridium thermocellum* batch cultures. Anal. Chem., 74, 219-227.

Zhang, Y-H.P., Ding, S-Y., Mielenz, J.R., Cui, J-B., Elander,R.T., Laser, M., Himmel, M.E., McMillan, J.R., Lynd, L.R. 2007. Fractionating recalcitrant lignocellulose at modest reaction conditions. Biotech. Bioeng., 97(2), 214-223.

Zhang, Y-H.P., Lynd, L.R. 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol. Bioeng., 88(7), 797-824.

Zhao, X., Cheng, K., Liu, D. 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl. Microbiol. Biot., 82(5), 815-827.

Zhu, Y.M., Lee, Y.Y., Elander, R.T. 2004. Dilute-acid pretreatment of corn stover using a high-solids percolation reactor. Appl. Biochem. Biotech., 117(2), 103-114.

Zverlov, V.V., Berezina, O., Velikodvorskaya, G.A., Schwarz, W.H. 2006. Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery. Appl. Microbiol. Biotechnol. 71(5), 587–597.

Appendix

16S rRNA gene sequence of culture BOH3

AGTGCGGCGTCTTACACATGCAGTCGAGCGATGAAGCTCCTTCGGGAGTG
GATTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTCATAGAGG
GGAATAGCCTTTCGAAAGGAAGATTAATACCGCATAAGATTGTAGTACCG
CATGGTACAGCAATTAAAGGAGTAATCCGCTATGAGATGGACCCGCGTCG
CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGAT
GCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTC
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TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACT
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GGCTTAACCTGGGTGCTGCATTCCAAACTGGATATCTAGAGTGCAGGAGA
GGAAAGGAGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAG
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GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
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CGAGAGTGAGCAAAACTATAAAACCGATCTCAGTTCGGATTGTAGGCTGA
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GGGAGTGGATTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTC
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CGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAA
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CAGGAGAGGAAAGGAGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGAT
TAGGAAGAATACCAGTGGCGAAGGCGCCTTTCTGGACTGTAACTGACACT
GAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGC
CGCCGCTAACGCATTAAGTATTCCGCCTGGGGAGTACGGTCGCAAGATTA
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGT

TTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGAA
TTACTCTGTAATGGAGGAAGCCACTTCGGTGGCAGGAAGACAGGTGGTGC
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GCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCACTCTAGCGAGA
CTGCCCGGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGC
CCCTTATGTCTAGGGCTACACACGTGCTACAATGGTCGGTACAATGAGAT
GCAACCTCGCGAGAGTGAGCAAAACTATAAAACCGATCTCAGTTCGGATT
GTAGGCTGAAACTCGCCTACATGAAGCTGGAGTTGCTAGTAATCGCGAAT
CAGAATTCGCGGAATG

Publications from this research work

Conferences

May 28th–30th, 2009: 1st Singapore-Hong Kong Bilateral Graduate Student Congress in Chemical Sciences (poster presentation). "Converting cellulose and hemicellulose to mono-sugars and hydrogen gas by an anaerobic bacterium", University Hall, National University of Singapore, Singapore

October 23th–24th, 2009: 2nd Regional Conference on Chemical Engineering, Chemical Engineering for Sustainable Development and Collaboration in the ASEAN Region (oral presentation). "Degradation of recalcitrant compounds by an anaerobic consortium", Ho Chi Minh City, Vietnam

March 23rd–25th, 2011: Bioenergy and Biorefinery Conference–South East Asia 2011 (oral presentation). "Biobutanol production from lignocellulosic biomass", Ngee Ann Polytechnic, Singapore

March 27th-31st, 2011: 241st ACS (American Chemical Society) National Meeting and Exposition (poster presentation). "Enhancing biobutanol production by manipulating an anaerobic microbe", Anaheim, California, the United States of America

October 16th–21st, 2011: AIChE (American Institute of Chemical Engineers) Annual Meeting 2011, Minneapolis, the United States of America (oral presentation). "Optimization of a wild-type mesophilic *Clostridium* species that produces butanol"

Peer-reviewed journals

Bramono, S.E., Lam, Y.S., Ong, S.L, He, J. 2011. A mesophilic *Clostridium* species that produces butanol from monosaccharides and hydrogen from polysaccharides. Bioresour. Technol., 102(20), 9558-9563.

"Optimization of butanol generation by *Clostridium* species strain BOH3".

(in preparation stage)

"Direct conversion of food waste to butanol by *Clostridium* species strain BOH3".

(in preparation stage)

Biography

Mr. Sandhi Eko Bramono was born in Jakarta, Indonesia, in 1980. He obtained his Bachelor of Environmental Engineering from Bandung Institute of Technology, Indonesia, in 2003. Straight after the completion of his bachelor degree, he pursued Master of Environmental Engineering and Science (by coursework) at the University of New South Wales (UNSW), Sydney, Australia, in 2003-2004. After finishing his master degree, he returned to Indonesia, and joined as an engineer for municipal solid and liquid waste treatment plants design with United Nations Development Programme (UNDP), in 2004-2006. In 2006, he pursued his PhD study in Department of Civil and Environmental Engineering, National University of Singapore (NUS), Singapore, under NUS research scholarship.